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THE BIOCHEMICAL JOURNAL

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CORRIGENDA

The separation and quantitative determination of the component sugars of mucoproteins

By A. Gottschalk and G. L. Ada

Volume 62 (1956)

p. 681, col. 1, line 22:

delete carbazole (Dische, Shettles & Osnos, 1949)

p. 681, col. 1, line 31:

for Dische (1947) *read* Dische, Shettles & Osnos (1949)

The Biochemistry of Aromatic Amines

2. THE CONVERSION OF ARYLAMINES INTO ARYLSULPHAMIC ACIDS AND ARYLAMINE-*N*-GLUCOSIDURONIC ACIDS*

By E. BOYLAND, D. MANSON and S. F. D. ORR
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(*Received* 20 *July* 1956)

Aromatic amines are known to be metabolized by conversion into acetamido derivatives and oxidation to aminophenols. The aminophenols may be conjugated or further metabolized. In studying the metabolism of 2-naphthylamine by paper chromatography two products were detected which gave the free amine on acidification (Boyland & Manson, 1955). One of these substances could be prepared by treating 2-naphthylamine with sodium glucuronate in aqueous solution and is presumably sodium 2-naphthylamine *N*-glucosiduronate. The other product was found to be 2-naphthylsulphamic acid; this product appears to be produced in greater amounts by rabbits than by rats and an analogous reaction occurs with aniline and 1-naphthylamine. Aliphatic sulphamate or *N*-sulphonate groups are probably present in naturally occurring polysaccharides such as heparin (Reinert & Winterstein, 1939). 2-Naphthylamine and other aromatic amines may be excreted as the *N*-glucosiduronic acids and as the *N*-sulphonic acids or sulphamates.

EXPERIMENTAL

Methods

Animals were kept in metabolism cages and the urine was collected daily. Rabbits (approx. 2·5 kg.) and guinea pigs (approx. 400 g.) were fed on cabbage, bran, rat cake and water, rats (approx. 200 g.) on rat cake, bread and water, and hamsters (approx. 85 g.) on rat cake, mash, mixed seeds and water. Amines were injected intraperitoneally in arachis oil. Rabbits were given 0·3 g. of aniline, 4-aminodiphenyl, 1-naphthylamine or 0·5 g. of 2-naphthylamine in 10 ml. of oil; rats received 0·03 g. of each of the first three amines and 0·05 g. of 2-naphthylamine in 1 ml. of oil; guinea pigs received 0·06 g. of 2-naphthylamine in 1·5 ml. of oil; hamsters received 0·02 g. of 2-naphthylamine in 0·5 ml. of oil.

Paper chromatography. Upward and downward development was employed with Whatman no. 1 chromatography paper. Solvent systems used were (*a*) *n*-butanol–propan-1-ol–0·1 N-NH_3 soln. (2:1:1, by vol.) (Booth, Boyland & Manson, 1955), (*b*) benzene–methanol–amyl alcohol (B.S. 696)–2 N-HCl (35:35:17·5:12·5, by vol.) (Ekman, 1948) and (*c*) *n*-butanol–acetic acid–water (2:1:1, by vol.).

Metabolites were detected on paper chromatograms by spraying with (1) N-HCl, then $NaNO_2$ (0·5%), followed by hexylresorcinol (0·5% in 2 N-NaOH) and (2) Ehrlich's reagent: *p*-dimethylaminobenzaldehyde (0·5% in ethanol containing 1 ml. of conc. HCl/100 ml.). R_F values and colour reactions are listed in Table 1.

Materials

Melting points are uncorrected. Aniline, 4-aminodiphenyl (*p*-xenylamine), 1-naphthylamine and 2-naphthylamine were from commercial sources.

Potassium 1-amino-2-naphthylsulphate and potassium 2-amino-1-naphthyl sulphate were prepared by oxidation of 1- and 2-naphthylamine respectively with potassium persulphate (Boyland, Manson & Sims, 1953), and potassium 4-amino-3-diphenylyl sulphate by oxidation of 4-aminodiphenyl with potassium persulphate (Boyland & Sims, 1954). Potassium 2-aminophenyl sulphate was prepared by reduction of potassium 2-nitrophenyl sulphate (Burkhardt & Wood, 1929).

Potassium salts of phenylsulphamic acid, 4-diphenylylsulphamic acid, 1-naphthylsulphamic acid and 2-naphthyl sulphamic acid were all prepared by treatment of the parent amines with chlorosulphonic acid in dry pyridine. Chlorosulphonic acid (5 g.) was added to pyridine (50 ml.) at 15°, followed by the amine (1/20 mole) and after keeping the mixture at room temperature overnight it was poured into 100 ml. of N-KOH. The solution was extracted several times with ether and the aqueous phase concentrated to low volume and cooled, when the compounds crystallized out as plates. They were recrystallized from aqueous ethanol: potassium phenylsulphamate (Found: N, 6·9. Calc. for $C_6H_6O_3NSK$: N, 6·6%); *potassium 4-diphenylylsulphamate* (Found: N, 4·8; S, 11·4. $C_{12}H_{10}O_3NSK$ requires N, 4·9; S, 11·15%); potassium 1-naphthylsulphamate (Found: N, 5·3. Calc. for $C_{10}H_8O_3NSK$: N, 5·4%); potassium 2-naphthylsulphamate (Found: N, 5·4; S, 12·5. Calc. for $C_{10}H_8O_3NSK$: N, 5·4; S, 12·3%).

Each gave a precipitate after slight warming with N-HCl and $BaCl_2$. All diazotized and coupled without prior hydrolysis but reacted only slowly with Ehrlich's reagent.

Dipotassium salt of the sulphuric ester of 1-hydroxy-2-naphthylsulphamic acid. This compound was prepared (1) by the action of chlorosulphonic acid on 2-amino-1-naphthol, and (2) by oxidation of potassium 2-naphthylsulphamate with potassium persulphate.

(1) 2-Amino-1-naphthol hydrochloride (3 g.) in water was treated with NaOH (1 equivalent) and $Na_2S_2O_4$ (0·5 g.) and the precipitated 2-amino-1-naphthol (plates) was washed with water and ether and dried over P_2O_5. The dry 2-amino-

* Part 1: Boyland, Manson, Sims & Williams (1956).

1-naphthol (2 g., m.p. 135–138° with decomposition) was added to carbon disulphide (50 ml.), dimethylaniline (10 ml.) and chlorosulphonic acid (1·4 g.). After standing overnight the solution was added to excess of 2N-KOH, extracted with ether and the aqueous phase evaporated to low volume. The *dipotassium salt of the sulphuric ester of 1-hydroxy-2-naphthylsulphamic acid* separated and was recrystallized from aqueous methanol (0·4 g., plates). (Found: N, 3·4; S, 16·5. $C_{10}H_7O_7NS_2K_2$ requires N, 3·5; S, 16·2%.) One equivalent only of chlorosulphonic acid had been employed in the hope of obtaining the unknown 1-hydroxy-2-naphthylsulphamic acid but this was not achieved. 2-Amino-1-naphthol hydrochloride gave predominantly 2-amino-1-naphthylsulphuric acid with chlorosulphonic acid (Boyland *et al.* 1953; and unpublished observations). (2) $K_2S_2O_8$ (7 g.) in water (75 ml.) was added to potassium 2-naphthylsulphamate (7 g.) and KOH (2·5 g.) in 50% aqueous acetone (250 ml.) over 1 hr. with stirring. After standing overnight, 2N-KOH (60 ml.) was added and the solution evaporated almost to dryness. The residue was extracted several times with a total of 150 ml. of hot methanol. After filtering and cooling the solution a mixture of product and starting material (1·5 g.) separated. Concentration to 100 ml. gave a further 1 g. of mixture and further concentration to 50 ml. gave 0·6 g. of almost pure product which after recrystallization from aqueous methanol gave the dipotassium salt of the sulphuric ester of 1-hydroxy-2-naphthylsulphamic acid. (Found: N, 3·6; S, 16·0. Calc. for $C_{10}H_7O_7NS_2K_2$: N, 3·5; S, 16·2%.) The concentration procedure and purification were guided by paper chromatography.

Products from methods (1) and (2) gave 2-amino-1-naphthol on boiling with dilute HCl, detected by the characteristic colours given when the solution was made alkaline with NH_3 soln. and extracted with benzene (Liebermann & Jacobson, 1882). The compound diazotized and coupled with hexylresorcinol to yield a mauve colour but reacted only slowly with Ehrlich's reagent. It could be run without decomposition in solvent system (*a*) but partially decomposed in solvent system (*c*) to yield 2-amino-1-naphthylsulphuric acid.

N-Glucosiduronic acids. The method of Smith & Williams (1949*b*) was adapted for the synthesis of 2-naphthylamine *N*-glucosiduronic acid as ammonium and sodium salts and for the preparation of a solution of 2-amino-1-naphthylsulphuric acid *N*-glucosiduronic acid. Analyses of the 2-naphthylamine *N*-glucosiduronic acid salts indicated that the compounds consisted of one molecule of amine and one molecule of *N*-glucosiduronic acid with the water of crystallization dependent on the derivative (cf. Smith & Williams, 1949*b*). *N*-Glucosides are also known to crystallize with water, alcohol or amine of crystallization (Mitts & Hixon, 1944).

Ammonium 2-naphthylamine N-glucosiduronate. D-Glucuronolactone (1 g.), dissolved in 0·3N-$Ba(OH)_2$ (20 ml.), was kept at room temperature for 3 hr. and then treated with $(NH_4)_2SO_4$ (saturated solution, 1 ml.). Barium sulphate was removed by centrifuging and the solution of ammonium glucuronate filtered (Ford's clarifying pad, grade F.C.B.). 2-Naphthylamine (freshly recrystallized, 1 g.) in ethanol (10 ml.) was added, and any precipitated amine removed by filtration and the filtrate allowed to stand for 1 hr. at room temperature, when the crystalline product (plates) was filtered off and washed successively with water, ethanol and

Table 1. *R_F values and colour reactions of some aromatic amines and derivatives*

All the compounds gave yellow colours with Ehrlich's reagent. For composition of solvent systems see text.

Compound	Fluorescence in ultraviolet light	Colour after diazotization and coupling with hexylresorcinol	R_F in solvent system (*a*) Descending	(*a*) Ascending	(*b*) Ascending	(*c*) Ascending
Aniline	None	Yellow	Volatilized	—	0·75	—
Phenylsulphamic acid	None	Yellow	0·33	—	Hydrolysis	—
2-Aminophenylsulphuric acid	None	Red	0·33	—	0·66	—
4-Aminodiphenyl	Faint blue	Yellow	0·96	—	0·93	—
4-Diphenylsulphamic acid	Faint blue	Yellow	0·40	—	Hydrolysis	—
4-Amino-3-diphenylylsulphuric acid	None	Red	0·47	—	0·70	—
1-Naphthylamine	Blue-white	Orange-yellow	0·94	—	0·90	—
1-Naphthylsulphamic acid	Blue	Orange-yellow	0·39	—	Hydrolysis	—
1-Amino-2-naphthylsulphuric acid	Blue-white	Blue	0·40	—	0·70	—
2-Naphthylamine	Blue-white	Yellow	0·94	0·95	0·90	0·98
2-Naphthylsulphamic acid	Blue	Yellow	0·40	0·55	Hydrolysis	0·68
2-Amino-1-naphthylsulphuric acid	Blue-white	Mauve	0·42	0·58	0·70	0·73
2-Naphthylamine-*N*-glucosiduronic acid	Blue-white	Yellow	—	0·28 (Partial hydrolysis)	—	Complete hydrolysis
2-Amino-1-naphthylsulphuric acid *N*-glucosiduronic acid	Blue-white	Mauve	0·08 (Partial hydrolysis)	0·15	—	0·30 (Partial hydrolysis)
1-Hydroxy-2-naphthylsulphamic acid sulphuric ester	Blue-white	Mauve	0·16	0·16	—	0·35 (Partial hydrolysis)

ether. Yield of *ammonium 2-naphthylamine N-glucosiduronate*, 1·2 g., m.p. 138–140° (decomp.). Recrystallization was difficult as prolonged heating with 50% ethanol caused decomposition, but a small amount was recrystallized for analysis, without altering the melting point. (Found: C, 61·9, 61·8; H, 6·45, 6·5; N, 8·4, 8·5. $C_{26}H_{29}O_6N_3,1{\cdot}5H_2O$ requires C, 61·75; H, 6·4; N, 8·3%.)

As the compound was insoluble in cold water, ethanol and pyridine the optical rotation could not be determined. It gave a rapid positive reaction for glucuronic acid with naphthoresorcinol solution. It was diazotized with HCl and $NaNO_2$ and the diazo compound coupled with hexylresorcinol. Cold 10N-NaOH liberated NH_3. On boiling with water the material decomposed and crystals of 2-naphthylamine (identified by m.p. and mixed m.p.) separated on cooling. On keeping in the dry state it became brown if exposed to daylight. Paper chromatography of the substance with solvent system (*a*) caused some decomposition, 2-naphthylamine and the *N*-glucosiduronic acid being detected. With a two-dimensional chromatogram further decomposition occurred during the second development.

Sodium 2-naphthylamine N-glucosiduronate. D-Glucuronolactone (1·7 g.) in water (10 ml.) was converted into sodium glucuronate by addition of NaOH (0·39 g.) and allowing the solution to stand at room temperature for 3 hr. An equal volume of a phosphate buffer (pH 7·0, 0·2 M) was then added followed by 2-naphthylamine (0·7 g.) in ethanol (50 ml.). The volume was made up to 100 ml. with water and the solution kept at 0° overnight. Elongated plates of *sodium 2-naphthylamine N-glucosiduronate* (0·5 g.) separated, the m.p., 191–192° (decomp.), being unchanged by recrystallization from aqueous ethanol.

$$[\alpha]_D^{23} - 115° \xrightarrow{24 \text{ hr.}} -25°$$

(value then constant). (*c*, 0·08 in water.) (Found: C, 62·0; H, 5·6; N, 6·0, 5·7; Na, 4·6, 4·8. $C_{26}H_{25}O_6N_2Na,H_2O$ requires C, 62·1; H, 5·4; N, 5·6; Na, 4·6%.)

Aqueous solutions of the compound became pinkish brown in colour after exposure to light.

Reaction of 2-amino-1-naphthylsulphuric acid with D-glucuronic acid. D-Glucuronolactone (1 g.) was converted into ammonium glucuronate as above and 2-amino-1-naphthylsulphuric acid (1·3 g., in aqueous ethanol, neutralized with dilute NH_3 soln.) was added. Paper chromatography showed the presence of a substance which diazotized and coupled with hexylresorcinol to give the same mauve colour as 2-amino-1-naphthylsulphuric acid. It had an R_F of 0·15 in solvent system (*a*) (ascending) and 0·3 in solvent system (*c*). The corresponding values for 2-amino-1-naphthylsulphuric acid were 0·58 (ascending) and 0·73. By analogy with the formation of 2-naphthylamine *N*-glucosiduronic acid this probably represented the corresponding derivative of the sulphuric ester. The spot believed to be the reaction product was cut out, re-attached to fresh paper and redeveloped in solvent system (*a*), when both the initial substance and 2-amino-1-naphthylsulphuric acid could be detected. The compound is thus unstable like 2-naphthylamine *N*-glucosiduronic acid. If the spot was sprayed with dilute HCl and heated at 70° for 5 min. before running again, only 2-amino-1-naphthylsulphuric acid was detectable after development of the chromatogram.

Attempts to isolate the compound were unsuccessful; gradual evaporation of the solution over P_2O_5 gave only ammonium 2-amino-1-naphthyl sulphate.

Infrared spectra

Infrared spectra of the samples as mulls in liquid paraffin or a fully fluorinated oil were measured on a Perkin–Elmer model 112 spectrometer, fitted with a NaCl prism.

RESULTS

2-Naphthylamine metabolism in rat, rabbit, guinea pig and hamster

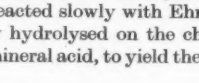
Aromatic amines are one of a group of compounds which cause excretion of large amounts of reducing material, probably mainly glucuronic acid (Bray, Hybs & Thorpe, 1951). 2-Naphthylamine produces the same effect and the glucuronic acid excreted might react with the amine and its metabolites to yield *N*-glucosiduronic acids. The reaction of *p*-toluidine with glucuronic acid has been described by Smith & Williams (1949*a*, *b*). Paper chromatography of urine from rats and rabbits injected with 2-naphthylamine showed that a spot of the same R_F and colour reactions as synthetic 2-naphthylamine *N*-glucosiduronic acid was present.

A two-dimensional chromatogram was carried out, being developed first in solvent system (*a*), and the paper dried and then sprayed with N-HCl along the line of the separated metabolites. The chromatogram was heated at 70° for 5 min. and dried. After treatment with ammonia vapour to neutralize residual acid (which causes distortion of the chromatogram) the sheet was developed in solvent system (*c*), which gives better definition of 2-naphthylamine than solvent system (*a*). 2-Naphthylamine was released from the spot corresponding to the *N*-glucosiduronic acid. The *N*-glucosiduronate could be detected by paper chromatography of urine immediately after it was passed. The urine was centrifuged and the deposit washed with water, ethanol and ether. This deposit gave a strong positive reaction with the Tollens test for glucuronic acid and chromatography showed the presence of the *N*-glucosiduronic acid. The presence of the compound in the deposit accords with the low solubility of the synthetic compounds. Guinea-pig and hamster urines were not examined for this compound.

The two-dimensional chromatogram described above also gave 2-naphthylamine from a spot (R_F 0·6 in the first development) which diazotized and coupled with hexylresorcinol to give a yellow colour. In addition, a spot having the same colour reactions as 2-amino-1-naphthylsulphuric acid but having an R_F value of 0·15 in solvent system (*a*) gave, by the above technique, 2-amino-1-naphthylsulphuric acid, R_F 0·73 in system (*c*).

Realization that the amino group of 2-naphthylamine could be substituted by a very acid-labile group suggested that 2-naphthylsulphamic acid

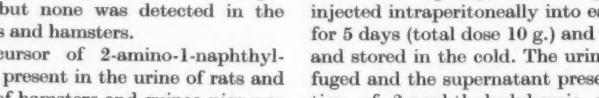
(I) might be a metabolite. This was found to behave in exactly the same manner as the compound with R_F 0·6, that is, although the amino group was substituted it could be readily diazotized and coupled and reacted slowly with Ehrlich's reagent. It was readily hydrolysed on the chromatograms, even by cold mineral acid, to yield the parent amine.



(I)

2-Naphthylsulphamic acid could be detected in rabbit urine without recourse to two-dimensional chromatography although the spot overlapped with 2-amino-1-naphthylsulphuric acid. Its presence was confirmed by its isolation from rabbit urine after administration of the amine. Only small amounts were present in rat urine, in which it could be detected only by the two-dimensional technique employed above, but none was detected in the urine of guinea pigs and hamsters.

The labile precursor of 2-amino-1-naphthylsulphuric acid was present in the urine of rats and rabbits (the urine of hamsters and guinea pigs was not examined for this metabolite) and might be a derivative in which the amino group is substituted (II) by glucuronic acid or (III) by sulphuric acid, or 1-hydroxy-2-naphthylsulphamic acid (IV) which could give 2-amino-1-naphthylsulphuric acid by migration of the sulphate group in the presence of acid.



(II) (III) (IV)

The precursor cannot be 2-acetamido-1-naphthylsulphuric acid as this has a different R_F value [0·63 in solvent system (*a*), upward development] and it has not been detected as a metabolite of 2-naphthylamine in rats or rabbits (unpublished observations).

The *N*-sulphonic acid and *N*-glucosiduronic acid of 2-amino-1-naphthylsulphuric acid have similar R_F values in the solvent systems employed (ascending or descending development). Both compounds yielded 2-amino-1-naphthylsulphuric acid on acid treatment of the chromatogram in the same way as the unknown metabolite, but the R_F value of the metabolite was closer to that of the *N*-glucosiduronic acid. 1-Hydroxy-2-naphthylsulphamic acid (IV) is not known and could not be synthesized. However, after treatment of 2-hydroxyphenylsulphamic acid with N-HCl no 2-aminophenylsulphuric acid could be detected so that migration of a sulphate ($-SO_3H$) group appears unlikely. If the spot due to the metabolite was cut out and extracted with methanol and the residue from the extract developed on a second chromatogram, or if the spot was cut out and run again after re-attachment to fresh paper, 2-amino-1-naphthylsulphuric acid could be detected. This decomposition on the chromatogram with the *n*-butanol–propan-1-ol–0·1 N-NH_3 soln. solvent system without any further treatment was characteristic of the synthetic *N*-glucosiduronic acid. Repeated running in this solvent system of a spot derived from the synthetic *ON*-disulphate (III) did not cause any decomposition although it is hydrolysed in acetic acid–butanol systems. This metabolite is therefore 2-amino-1-naphthylsulphuric acid *N*-glucosiduronic acid. The corresponding spot in rabbit urine behaved in the same way.

Isolation of sodium 2-naphthylamine N-glucosiduronate. 2-Naphthylamine (0·5 g. in arachis oil) was injected intraperitoneally into each of four rabbits for 5 days (total dose 10 g.) and the urine collected and stored in the cold. The urine (2 l.) was centrifuged and the supernatant preserved for the isolation of 2-naphthylsulphamic acid (below). The deposit was washed three times with 10 ml. portions of water, flocculent material being decanted, and then washed twice with ethanol and twice with ether. The deposit was then extracted twice with hot (60°) 50% ethanol, and the extract filtered and concentrated to dryness in a desiccator at room temperature. The residue gave a rapid positive Tollens reaction. Paper chromatography showed the presence of a substance identical with synthetic 2-naphthylamine *N*-glucosiduronic acid. After three crystallizations from 50% ethanol to remove amorphous and inorganic impurities, crystalline material (1·5 mg.) was obtained, m.p. 180–185°.

Infrared spectral analysis (Fig. 1) also indicated that the substance was identical with synthetic sodium 2-naphthylamine *N*-glucosiduronate, except for some slight further absorption in the isolated sample at 1045 and 1115 cm.$^{-1}$; this must be due to the presence of an impurity in the isolated sample.

Isolation of sodium 2-naphthylsulphamate. The supernatant from the isolation of the *N*-glucosiduronic acid was filtered and passed through a charcoal column (approx. 150 g. of activated

charcoal, British Drug Houses Ltd.) under slight suction. The eluate was almost colourless and showed no detectable naphthylamine metabolites. The charcoal was then washed with water (1·5 l.). This eluate also contained no detectable metabolites. Hot methanol was then passed through the column in six 500 ml. portions, which were collected separately and examined by paper chromatography. Some separation of 2-amino-1-naphthylsulphuric acid from 2-naphthylsulphamic acid occurred as the first and second fractions contained more of the sulphuric ester than the third and fourth, which were richer in the sulphamic acid derivative. The fifth and sixth fractions contained much less of both compounds and the sixth contained 2-acetamido-6-naphthylglucosiduronic acid (isolation of this compound will be described separately). The third, fourth and fifth fractions were combined, distilled to dryness after making alkaline with 2N-NaOH, and the gummy residue was extracted several times with hot methanol to separate inorganic material. The combined methanol extracts were evaporated to dryness, the residue was dissolved in the minimum of *n*-butanol–propan-1-ol–water (2:1:1, by vol.) and Whatman cellulose powder added to form a paste. The paste was added to the top of a cellulose column (23 cm. × 5 cm.) prepared in the same solvent mixture and the column developed over 18 hr., the eluate being collected in 50 ml. portions. Paper chromatography showed that the first two fractions contained 2-acetamidonaphthalene, 2-acetamido-6-naphthol and a compound believed to be 2-acetamido-5:6-dihydroxy-5:6-dihydronaphthalene. The third, fourth and fifth fractions contained 2-amino-1-naphthylsulphuric acid and 2-naphthylsulphamic acid and were evaporated to dryness and the residue was crystallized from 70% ethanol. After cooling overnight crystals (plates) of almost pure sodium 2-naphthylsulphamate were deposited which were recrystallized from 70% ethanol. Yield, 0·023 g. (Found: N, 5·7; S, 13·1. Calc. for $C_{10}H_8O_3NSNa$: N, 5·7; S, 13·1%.) In another experiment 0·019 g. of sodium 2-naphthylsulphamate was isolated after injection of 6 g. of 2-naphthylamine. The R_F value was identical with that of the synthetic compound, and it could be diazotized and coupled with hexylresorcinol to give a yellow colour without prior hydrolysis, but it reacted slowly with Ehrlich's reagent. The compound yielded inorganic sulphate upon warming with dilute HCl. It gave a persistent yellow colour to a Bunsen flame. Hydrolysis of the compound with dilute HCl, and evaporation of the solution and treatment of the residue with benzoyl chloride in pyridine, gave 2-benzamidonaphthalene, m.p. 160–163° with no depression of m.p. with an authentic specimen of 2-benzamidonaphthalene.

Concentration of the mother liquors of the first experiment gave only mixtures of sodium 2-amino-1-naphthyl sulphate and 2-naphthylsulphamate, which could not be separated. Cellulose-column chromatography of the material in the first and second methanol washings of the charcoal column gave mainly sodium 2-amino-1-naphthyl sulphate. To obtain the sulphuric ester all these fractions were combined and acidified with dilute HCl. The precipitate was collected and recrystallized by solution in dilute alkali and precipitation with dilute acid to yield 2-amino-1-naphthylsulphuric acid (0·049 g.), m.p. 224–226°, which gave no depression of melting point with an authentic specimen. The second experiment gave 0·029 g. of the ester. 2-Amino-1-naphthylsulphuric acid has not previously been isolated from urine of rabbits injected with 2-naphthylamine.

Aniline metabolism in rabbits and rats

Phenylsulphamic acid could not be separated from 2-aminophenylsulphuric acid by paper chromatography in solvent system (*a*) or (*c*). The sulphuric ester was present in both species after administration of the amine. Aniline could not be detected after development with solvent system (*a*) or (*c*) owing to its volatility. Hence phenylsulphamic acid could not be identified (as aniline) after two-dimensional chromatography with an intermediate acid hydrolysis as described for 2-naphthylsulphamic acid. After application of the urine the chromatogram was developed first in solvent system (*a*) for 18 hr. and then with solvent system (*b*) for 6 hr., when phenylsulphamic acid is hydrolysed but 2-aminophenylsulphuric acid is not. After treatment of rabbit urine in this manner aniline was detectable, released from a spot with an R_F value corresponding to that of phenylsulphamic acid. Phenylsulphamic acid could not be detected in rat urine.

4-*Aminodiphenyl metabolism in rabbits and rats*

4-Diphenylylsulphamic acid and 4-amino-3-diphenylylsulphuric acid were separable by paper chromatography in solvent system (*a*). No sulphamic acid derivative was detected in rat or rabbit urine with this solvent system or by two-dimensional chromatography in this solvent system followed by solvent system (*b*).

1-*Naphthylamine metabolism in rabbits and rats*

1-Naphthylsulphamic acid was detected in rabbit urine by the method used for the detection of 2-naphthylsulphamic acid. None was detected in rat urine.

Infrared-absorption spectra of 2-naphthylamine and the carbohydrate derivatives

The main features of the several spectra (Fig. 1) are the sharp bands between 750 and 850 cm.$^{-1}$ due to the out-of-plane deformation modes of the aromatic CH groups, the strong absorption at 950–1100 cm.$^{-1}$ in the sugar derivatives, the pair of very strong bands at about 1400 and 1600 cm.$^{-1}$ due to the carboxylic ion in the *N*-glucosiduronates and the strong absorption at about 1450 cm.$^{-1}$ due to the ammonium ion.

The band at about 1630 cm.$^{-1}$, present in 2-naphthylamine itself, persists in each of the sugar derivatives. Although partly due to the amino group in the amine, it must also be connected with the CC vibrations of the naphthalene nucleus, and a band at this position is present in the spectrum of 2-naphthol. If the aromatic nucleus were conjugated with a N=C bond, this band should be shifted to lower frequencies, and another band at about 1660 cm.$^{-1}$ would be expected (Bellamy, 1954*a*). The persistence of the band at 1630 cm.$^{-1}$ therefore suggests the presence of the –NH-C$\leqslant$ structure in each of the sugar derivatives. The similarity of their absorption at 745, 810 and 836 cm.$^{-1}$, due to the CH deformation modes, confirms this similarity in structure. No band due to the NH group itself could be assigned with certainty; the stretching vibration at 3 μ. is overlapped with absorption due to the sugar hydroxyl groups, and the deformation vibration does not show any well-defined characteristic absorption band (Bellamy, 1954*b*).

DISCUSSION

Arylsulphamic acids appear to be a new type of arylamine metabolite; these are stable in neutral solution but hydrolysed by cold acid.

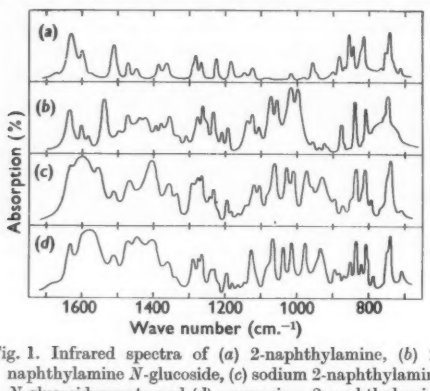


Fig. 1. Infrared spectra of (*a*) 2-naphthylamine, (*b*) 2-naphthylamine *N*-glucoside, (*c*) sodium 2-naphthylamine *N*-glucosiduronate and (*d*) ammonium 2-naphthylamine *N*-glucosiduronate.

Bushby & Woiwod (1955, 1956) have described the isolation of an *N*-glucosiduronic acid of 4:4′-diaminodiphenylsulphone from rabbit urine after administration of the parent amine. The excretion of labile glucosiduronic acids was proposed by Smith & Williams (1949*a*, *b*) to account for the presence of free glucuronic acid in the glucosiduronic acid fractions of aniline, *p*-phenetidine, *o*-, *m*- and *p*-anisidine urines. They considered the excretion of free glucuronic acid unlikely though not impossible. Bray *et al.* (1951) have shown, however, that reducing material, probably glucuronic acid, is present in rabbit urine after administration of several aromatic compounds. Some of this must be free and not conjugated, because the amount produced is too great to be accounted for by the dose of foreign compound administered, and the excretion continues after cessation of dosing. The formation of the *N*-glucosiduronic acid may occur in the urine by combination of the free amine and glucuronic acid after excretion rather than in the body tissues, but this would seem to be unlikely, particularly as Bushby & Woiwod (1956) found evidence of an *N*-glucosiduronic acid present in the blood of rabbits.

Booth *et al.* (1955) reported the formation of *N*-glucosides as artifacts during a study of the metabolism of 2-naphthylamine by rat-liver and kidney slices. The compounds arose by reaction of the amine and its derivatives with glucose in the Ringer solution employed.

A practical point in paper chromatographic analysis arising from the identification of *N*-sulphonic and *N*-glucosiduronic acids is that substances which diazotize and couple on chromatograms are not necessarily free aromatic amines. This may have some importance in the identification of naturally occurring aromatic amines.

SUMMARY

1. The urine of rabbits dosed with 2-naphthylamine contains two acid-labile derivatives of 2-naphthylamine: 2-naphthylsulphamic acid and 2-naphthylamine *N*-glucosiduronic acid.

2. Sodium 2-naphthylsulphamate, which readily yields 2-naphthylamine in cold acid solution, has been isolated from the urine of rabbits and detected by paper chromatography in the urine of rats but not of hamsters or guinea pigs dosed with 2-naphthylamine.

3. Phenylsulphamic acid was detected in urine of rabbits but not of rats dosed with aniline. 1-Naphthylsulphamic acid was detected in the urine of rabbits but not of rats dosed with 1-naphthylamine. 4-Diphenylylsulphamic acid did not appear to be formed from 4-aminodiphenyl by either rats or rabbits.

4. Sodium 2-naphthylamine *N*-glucosiduronate was isolated from urine of rabbits dosed with 2-naphthylamine and detected in urine of rats dosed with the amine.

5. The 2-naphthylamine *N*-glucosiduronate was shown by infrared absorption to have a structure similar to 2-naphthylamine *N*-glucoside.

6. Urine of rats and rabbits dosed with 2-naphthylamine contains an acid-labile derivative of 2-amino-1-naphthylsulphuric acid. This compound is not the sulphuric ester of 1-hydroxy-2-naphthylsulphamic acid, which was synthesized, but is 2-amino-1-naphthylsulphuric acid *N*-glucosiduronic acid.

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Glutathione Metabolism

2. THE OXIDATION AND REDUCTION OF GLUTATHIONE IN INTACT ERYTHROCYTES*

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The sulphydryl–disulphide oxidation–reduction system is involved in a large number of biological processes. The metabolic activity of many of these processes is dependent, to a large extent, on the level of oxidation of the sulphur constituent. The glutathione system (GSH⇌GSSG), as the chief soluble sulphydryl–disulphide component of the cell, is thought to play an important role in the regulation of the oxidation–reduction equilibrium of the cell by virtue chiefly of its effect on other sulphydryl–disulphide systems (see Barron, 1951). Thus reduced glutathione has been found to activate or protect sulphydryl enzymes or coenzymes under a variety of conditions, whereas a number of reactions dependent on an intact disulphide group are inhibited by this substance. Conversely, oxidized glutathione is inhibitory to many sulphydryl systems. The relative proportion of reduced to oxidized glutathione within the cell, as well as the factors which control this steady state, may therefore have an important influence on the regulation of intracellular metabolism.

The oxidation and reduction of glutathione by cell-free tissue preparations has been extensively investigated (see Vennesland & Conn, 1954). Less is known, however, of the factors which control the SS–SH equilibrium within the intact cell. As an approach to this problem, an investigation of the oxidation and reduction of glutathione in the intact erythrocyte was undertaken.

The exposure of intact erythrocytes to oxygen leads to a fall in the reduced glutathione level (Meldrum, 1932). In addition, the glyoxalase activity of the cells is decreased (Jowett & Quastel, 1933). Since reduced glutathione is a specific cofactor in the glyoxalase system (Lohmann, 1932), it was suggested (Jowett & Quastel, 1933) that the

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inhibition of glyoxalase activity by oxygen is a result of the oxidation of glutathione. The inhibition of glyoxalase activity by oxygen is prevented by the addition of glucose (Jowett & Quastel, 1933). The fall in reduced glutathione level is similarly prevented (Meldrum, 1932), which suggests that, in the intact erythrocyte, the glyoxalase activity is limited by the reduced glutathione level and therefore may be used for an investigation of the factors affecting the oxidation and reduction of glutathione in the intact cell. A study of the properties of the glyoxalase system of intact and lysed erythrocytes has been previously reported (Klebanoff, 1956).

METHODS

Collection and preparation of erythrocytes. Human blood was collected from the antecubital fossa with a syringe containing one drop of heparin (Connaught, 1000 units/ml.), care being taken to avoid haemolysis. The blood was immediately centrifuged at 3000 *g* for 10 min. and the plasma and buffy coat were removed. The erythrocytes were washed three times with 0·16M-NaCl, and diluted with saline to double the packed cell volume. The cells were freshly prepared once a week and stored at 5°.

Estimation of glyoxalase activity. The glyoxalase activity of intact erythrocytes was determined manometrically as previously described (Klebanoff, 1956). The flasks were gassed with either $CO_2 + N_2$ (5:95) or $CO_2 + O_2$ (5:95). The intact erythrocyte proved to be ideal for the measurement of glyoxalase activity in the presence of O_2, since under the conditions employed (0·05 ml. of the erythrocyte preparation in a total volume of 2·0 ml.), the O_2 uptake and the CO_2 release in the absence of methylglyoxal were negligible. The results reported are representative of a number of experiments.

Estimation of haemolysis. Since, in the absence of added glutathione, lysis of the erythrocytes results in an almost complete loss of glyoxalase activity (Jowett & Quastel, 1933), the degree of haemolysis was determined after the completion of each glyoxalase estimation. The contents of the Warburg vessels were centrifuged at 3000 *g* for 10 min. A volume (1·2 ml.) of the supernatant solution was added to 4·8 ml. of 0·1M-Na_2CO_3, the whole mixed and the intensity of colour determined in an EEL portable colorimeter (Evans Electroselenium Ltd., Harlow, Essex) with a Chance OGR 1 green filter. The percentage of haemolysis was estimated by comparison with a standard curve.

Chemical estimation of reduced glutathione. A volume (1·0 ml.) of the erythrocyte preparation, the reactants under investigation and 0·16M-NaCl to a final volume of 1·5 ml. were placed in duplicate Thunberg tubes. The tubes were evacuated and filled with gas as indicated. The reagents were mixed in a mechanical shaker at room temperature for a specified period and the glutathione level was estimated by the cyanide–nitroprusside method of Grunert & Phillips (1951).

RESULTS

Effect of plasma

The inhibitory effect of O_2 on the glyoxalase system of intact erythrocytes became progressively more pronounced on storage of the cells at 5° for periods up to 2 weeks. Further, cells well washed with saline were more susceptible to the inhibitory effect of O_2 than were poorly washed or unwashed cells. The glyoxalase activity of aerated cells supplemented with small amounts of plasma was greater than that of cells incubated with O_2 in the absence of plasma (Fig. 1). The protective effect of plasma could be more readily demonstrated by the use of cells stored at 4° for several days, as a result of the more pronounced inhibition of glyoxalase activity by O_2 after storage. The effect of plasma was maximal at concentrations as low as 1·25% of the total flask contents, a further increase in plasma concentration to 15% producing no further protection. At concentrations higher than 15%, however, the protective effect of plasma appeared to be less complete. The glyoxalase activity as indicated by the evolution of CO_2 fell, the effect being more pronounced as the plasma concentration was increased. The inhibition of glyoxalase activity was not

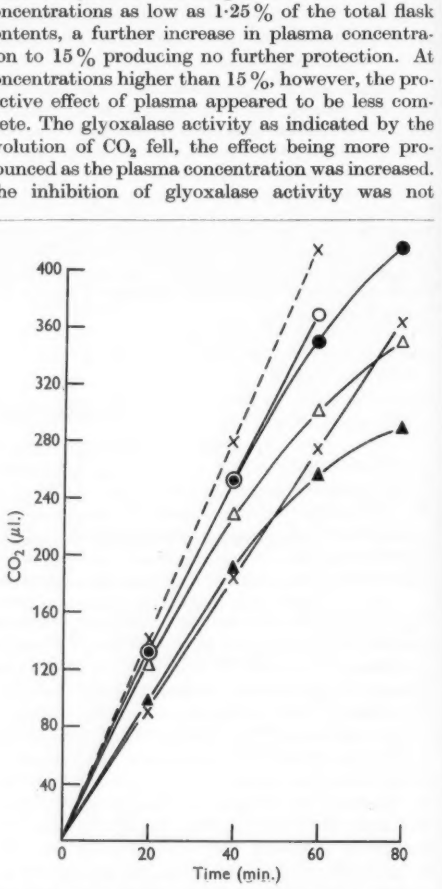

Fig. 1. Effect of plasma on the glyoxalase activity of aerated erythrocytes. The main vessel contained 0·05 ml. of the erythrocyte preparation, 0·4 ml. of 0·2M-$NaHCO_3$ and plasma as follows: none (×); 0·025–0·3 ml. (○); 0·4 ml. (●); 0·6 ml. (△); 1·2 ml. (▲); and 0·16M-NaCl in a final volume of 2·0 ml. The side arm contained 0·2 ml. of 1% methylglyoxal. The gas phase was either $CO_2 + N_2$ (5:95), – – –, or $CO_2 + O_2$ (5:95),——.

associated with a corresponding increase in haemolysis.

The exposure of erythrocytes to O_2 resulted also in a fall in reduced glutathione level which was prevented by the addition of plasma (Table 1).

Effect of glucose

The protective effect of plasma was unaltered by previous exposure to 60° for periods up to 60 min., indicating that the factor or factors responsible were heat-stable. A protein-free ultrafiltrate of fresh human plasma also protected the glyoxalase system from the inhibitory effect of O_2, although not to the same extent as did fresh whole plasma. Thus plasma components of low molecular weight were implicated. Glucose increased the glyoxalase activity of aerated erythrocytes in concentrations as low as 10^{-5} M, the effect reaching a maximum at a concentration of approximately 10^{-3} M (Table 2, Expt. *A*). The effect of glucose was less evident at concentrations above 10^{-2} M, whereas a distinct inhibition of glyoxalase activity resulted from the presence of glucose at a concentration of 16×10^{-2} M. However, glucose in concentrations greater than 10^{-2} M also produced haemolysis of the erythrocytes. The decline in glyoxalase activity and the haemolysis were completely prevented by an increase in the concentration of the NaCl from isotonic to hypertonic levels (Table 2, Expt. *B*). Furthermore, the protective effect of glucose on the glyoxalase activity of the aerated cells was again evident.

Mannose, fructose and, to a lesser extent, galactose and maltose were also found to protect the glyoxalase system of intact erythrocytes from the inhibitory effect of O_2 although they were less effective than glucose in this regard. Lactose, sucrose and D- and L-arabinose had no effect. The stimulatory effect of the various sugars on the glyoxalase activity of aerated cells was not additive with that of plasma, nor did a combination of the sugars give a greater activity than glucose alone. The stimulation produced by plasma was greater than that produced by an equivalent concentration of glucose. The addition of glucose, mannose, fructose, galactose and maltose to aerated erythrocytes also resulted in an increase in the reduced glutathione level (Meldrum, 1932; see also Table 1).

Table 1. *Effect of various substances on the reduced glutathione (GSH) level of erythrocytes*

Procedure as described in Methods, with supplements and gas phase as indicated. Incubation period, 6 hr. Figures for GSH are mg./100 ml. of cell suspension in saline (1:1, v/v).

Supplement (final concn.)	Gas phase	GSH (mg. %)
None	$CO_2 + N_2$ (5:95)	30·0
None	N_2	29·0
None	$CO_2 + O_2$ (5:95)	19·0
None	O_2	9·0
Plasma (33 %)	O_2	36·0
Glucose (5×10^{-3} M)	O_2	35·0
Adenosine ($1{\cdot}7 \times 10^{-3}$ M)	O_2	34·0
Inosine ($1{\cdot}7 \times 10^{-3}$ M)	O_2	34·0
Bovine albumin (25 mg.–2 g./100 ml.)	O_2	9·0–12·0

Effect of adenosine and inosine

The metabolism of adenosine by the intact erythrocyte is associated with an increase in the phosphate exchange (Prankerd & Altman, 1954) and in the glucose uptake (Gabrio, Hennessey, Thomasson & Finch, 1955), and leads to the expulsion of sodium by the cell (Harris & Prankerd, 1955) as well as to the maintenance of the intracellular potassium level (Gabrio, Donohue & Finch, 1955). Adenosine, in concentrations as low as 10^{-5} M, also produced a distinct increase in the glyoxalase activity of aerated cells (Table 3). Inosine produced a stimulatory effect similar in magnitude to that of adenosine. The effect of adenosine at maximal concentrations was not additive with that of glucose at maximal concentrations, although a summation of

Table 2. *Effect of glucose on the glyoxalase activity of aerated erythrocytes*

The main vessel contained 0·05 ml. of the erythrocyte preparation, 0·4 ml. of 0·2 M-$NaHCO_3$, and NaCl and glucose at the final concentrations indicated. The side arm contained 0·2 ml. of 1 % methylglyoxal. The final volume was 2·0 ml. and the gas phase $CO_2 + O_2$ (5:95). Erythrocyte preparation was 2 days old.

Expt.	Glucose (M)	Sodium chloride (M)	Tonicity	Glyoxalase activity: μl. of CO_2/60 min.	Glyoxalase activity: Difference (%)	Haemolysis (%)
A	—	$10{\cdot}8 \times 10^{-2}$	Isotonic	290	—	6
	$3{\cdot}3 \times 10^{-4}$	$10{\cdot}8 \times 10^{-2}$	Isotonic	350	+21	6
	$3{\cdot}3 \times 10^{-3}$	$10{\cdot}8 \times 10^{-2}$	Isotonic	348	+20	6
	$1{\cdot}6 \times 10^{-2}$	$10{\cdot}0 \times 10^{-2}$	Isotonic	331	+14	15
	$8{\cdot}2 \times 10^{-2}$	$6{\cdot}8 \times 10^{-2}$	Isotonic	300	+ 3	35
	$16{\cdot}5 \times 10^{-2}$	$2{\cdot}8 \times 10^{-2}$	Isotonic	232	−20	80
B	$16{\cdot}5 \times 10^{-2}$	$3{\cdot}7 \times 10^{-2}$	Hypertonic	306	+ 6	42
	$16{\cdot}5 \times 10^{-2}$	$6{\cdot}2 \times 10^{-2}$	Hypertonic	350	+21	14
	$16{\cdot}5 \times 10^{-2}$	$11{\cdot}2 \times 10^{-2}$	Hypertonic	384	+32	5

effects was evident when submaximal concentrations of each were used. D-Ribose and adenine were without effect on the glyoxalase system. The fall in reduced glutathione level which results from the aeration of erythrocytes was also prevented by the addition of adenosine or inosine (Table 1).

Effect of albumin

A number of tissue preparations are known to exist in a better functional state *in vitro* in the presence of serum proteins. Thus the surviving rat diaphragm has a higher rate of glucose uptake when incubated in a solution containing 3 % of bovine albumin than in the absence of albumin (Bornstein & Park, 1953). As can be seen in Table 4, the addition of bovine albumin (Armour), in concentrations as low as 25 mg. %, produced an increase in the glyoxalase activity of the aerated cells. An increase in reduced glutathione level was not observed under comparable conditions (Table 1). An increase in glyoxalase activity was produced by serum albumin in the presence of optimum concentrations of glucose or adenosine, whereas no further effect was observed in the presence of optimum concentrations of plasma. As with large concentrations of plasma (Fig. 1), albumin in concentrations greater than 1 g. % produced an inhibition of glyoxalase activity (Fig. 2).

Table 3. *Effect of adenosine and inosine on the glyoxalase activity of aerated erythrocytes*

Conditions as in Table 2 except for the addition of supplements as indicated. Erythrocyte preparation was 3 days old. Gas phase $CO_2 + O_2$ (5:95).

	Glyoxalase activity	
Supplement	μl. of CO_2/60 min.	Difference (%)
None	205	—
Adenosine (10^{-5} M)	245	+19
Adenosine (10^{-4} M)	270	+31
Adenosine (10^{-3} M)	266	+30
Inosine (10^{-5} M)	240	+17
Inosine (10^{-4} M)	265	+29
Inosine (10^{-3} M)	268	+31

Table 4. *Effect of bovine albumin on the glyoxalase activity of aerated erythrocytes*

Conditions as in Table 2 except for the addition of supplements as indicated. Erythrocyte preparation was 2 days old. Gas phase $CO_2 + O_2$ (5:95).

	Glyoxalase activity	
Supplement	μl. of CO_2/60 min.	Difference (%)
None	305	—
Albumin (25 mg. %)	338	+11
Albumin (100 mg. %)	346	+13
Albumin (250 mg. %)	375	+23
Albumin (1 g. %)	376	+24

The decrease in glyoxalase activity in the presence of large concentrations of plasma (Fig. 1) or bovine albumin (Fig. 2) was observed under both aerobic and anaerobic conditions and was not associated with a decrease in the reduced glutathione level of the cells. The further addition of methylglyoxal to the 'inhibited' preparation led to a return of activity (Fig. 2), suggesting that the fall in CO_2 output is not due to an inhibition of the enzyme system but to a decrease in the substrate concentration. This is supported by further evidence. The pre-incubation of the erythrocyte preparation with large concentrations of plasma for periods varying from 10 to 60 min. did not alter the response of the cells to the subsequent addition of methylglyoxal. The fall in CO_2 output always occurred after a lag period of similar length. This is contrary to the expected response if an inhibitory

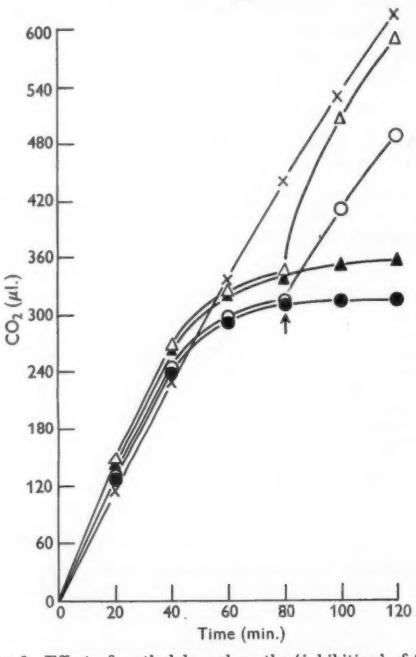

Fig. 2. Effect of methylglyoxal on the 'inhibition' of the glyoxalase activity by plasma and bovine albumin. The main vessel of double-side-arm Warburg flasks contained 0·05 ml. of the erythrocyte preparation, 0·4 ml. of 0·2 M-$NaHCO_3$, 1·2 ml. of plasma (△, ▲), 0·8 ml. of bovine albumin (0·1 g./ml.) (○, ●) and 0·16 M-NaCl in a final volume of 2·0 ml. One side arm contained 0·2 ml. of 1 % methylglyoxal, which was added at zero time. The other side arm contained 0·2 ml. of 0·16 M-NaCl (×, ●, ▲) or 0·2 ml. of 1 % methylglyoxal (○, △) which was added at the arrow. The gas phase was $CO_2 + N_2$ (5:95).

principle had been in contact with the erythrocytes during the pre-incubation period. Furthermore, the the level of CO_2 output at which the decline in glyoxalase activity occurred was found to vary directly with the methylglyoxal concentration. The formation of a complex between methylglyoxal and various chemical groups, e.g., sulphydryl groups (Schubert, 1935) of the plasma proteins, may account for the decrease in glyoxalase activity.

Effect of glucosamine

Harpur & Quastel (1949) demonstrated the phosphorylation of glucosamine by ATP in acetone-dried brain extracts. Glucose and fructose appeared to compete with glucosamine for phosphorylation by this tissue, with glucose having the greatest affinity. Glucosamine 6-phosphate formed under the influence of hexokinase was found to inhibit the glucokinase and glycolysis of *Schistosoma mansoni* (Bueding & MacKinnon, 1955) and to inhibit competitively the glucose 6-phosphate dehydrogenase system of yeast (Glaser & Brown, 1955). The intracellular transfer of glucosamine in the eviscerated and nephrectomized rabbit is under the influence of insulin and is associated with a substantial decrease in the transfer rate of glucose (Wick, Drury, Nakada, Barnet & Morita, 1955). Glucosamine similarly inhibits the uptake of glucose by the isolated rat diaphragm (Nakada, Morita & Wick, 1955).

In view of the inhibitory effect of glucosamine on glucose metabolism, this substance was tested on the glyoxalase system of intact erythrocytes. Glucosamine hydrochloride (Eastman), neutralized with NaOH, at concentrations of $0{\cdot}4$–$1{\cdot}6 \times 10^{-2}$M produced an inhibition of glyoxalase activity although haemolysis was negligible (Table 5). An increase in the concentration of glucosamine (4–8×10^{-2}M) produced considerable haemolysis, which was almost completely prevented by an increase in the NaCl concentration from isotonic to sufficiently hypertonic levels. The addition of NaCl, however, had little or no effect on the inhibition of glyoxalase activity. The further addition of methylglyoxal or sodium bicarbonate to the inhibited preparation did not alter the glyoxalase activity.

The inhibition of the glyoxalase activity by glucosamine was partially reversed by the addition of plasma, glucose, adenosine or inosine (Table 6). Glucose, at concentrations greater than $1{\cdot}6 \times 10^{-2}$M, caused a further decrease in glyoxalase activity associated with an increase in haemolysis. The

Table 5. *Effect of glucosamine on the glyoxalase activity of aerated erythrocytes*

Conditions as in Table 2, except for the addition of glucosamine and NaCl as indicated. Blood was 1 day old. Gas phase $CO_2 + O_2$ (5:95).

Expt.	Glucosamine (M)	Sodium chloride (M)	Tonicity	Glyoxalase activity: µl. of CO_2/60 min.	Glyoxalase activity: Difference (%)	Haemolysis (%)
A	—	$10{\cdot}8 \times 10^{-2}$	Isotonic	346	—	3
	$0{\cdot}4 \times 10^{-2}$	$10{\cdot}8 \times 10^{-2}$	Isotonic	325	− 6	4
	$0{\cdot}8 \times 10^{-2}$	$10{\cdot}8 \times 10^{-2}$	Isotonic	289	−16	6
	$1{\cdot}6 \times 10^{-2}$	$9{\cdot}2 \times 10^{-2}$	Isotonic	236	−32	7
	$4{\cdot}0 \times 10^{-2}$	$7{\cdot}6 \times 10^{-2}$	Isotonic	139	−60	55
	$8{\cdot}0 \times 10^{-2}$	$2{\cdot}8 \times 10^{-2}$	Isotonic	81	−77	73
B	$8{\cdot}0 \times 10^{-2}$	$5{\cdot}3 \times 10^{-2}$	Hypertonic	80	−78	60
	$8{\cdot}0 \times 10^{-2}$	$7{\cdot}8 \times 10^{-2}$	Hypertonic	86	−75	25
	$8{\cdot}0 \times 10^{-2}$	$12{\cdot}8 \times 10^{-2}$	Hypertonic	85	−75	6

Table 6. *Effect of glucose, adenosine and inosine on the inhibition of glyoxalase activity by glucosamine*

Conditions as in Table 2, except for the addition of glucosamine and supplements as indicated. Glucosamine was added to a final concn. of $1{\cdot}6 \times 10^{-2}$M in all cases except the first, the control, in which no glucosamine was present. Erythrocyte preparation was 2 days old. Gas phase $CO_2 + O_2$ (5:95).

Sodium chloride (M)	Glucosamine	Supplement	Tonicity	Glyoxalase activity: µl. of CO_2/60 min.	Glyoxalase activity: Difference (%)	Haemolysis (%)
$10{\cdot}8 \times 10^{-2}$	—	—	Isotonic	276	—	8
$9{\cdot}2 \times 10^{-2}$	+	—	Isotonic	177	−36	19
$7{\cdot}6 \times 10^{-2}$	+	Plasma (10%)	Isotonic	213	−23	20
$8{\cdot}4 \times 10^{-2}$	+	Glucose ($0{\cdot}16 \times 10^{-2}$M)	Isotonic	210	−24	22
$5{\cdot}2 \times 10^{-2}$	+	Glucose (8×10^{-2}M)	Isotonic	154	−44	88
$1{\cdot}2 \times 10^{-2}$	+	Glucose (16×10^{-2}M)	Isotonic	104	−62	95
$11{\cdot}2 \times 10^{-2}$	+	Glucose (16×10^{-2}M)	Hypertonic	225	−18	15
$20{\cdot}4 \times 10^{-2}$	+	—	Hypertonic	180	−35	6
$9{\cdot}2 \times 10^{-2}$	+	Adenosine (10^{-2}M)	Isotonic	203	−26	36
$9{\cdot}2 \times 10^{-2}$	+	Inosine (10^{-2}M)	Isotonic	207	−25	34

Table 7. *Effect of glucosamine on the reduced glutathione (GSH) level of erythrocytes*

Conditions as in Table 1, except that the tonicity of the control and of the experimental tubes was maintained at the same level by the use of hypertonic NaCl solutions. Figures for GSH are mg./100 ml. of cell suspension in saline (1:1, v/v).

Supplement	Gas phase	GSH (mg. %)
None	N_2	29·3
Glucosamine (0·06 M)	N_2	28·2
Glucosamine (0·15 M)	N_2	23·7
Glucosamine (0·3 M)	N_2	15·0
None	O_2	16·5
Glucosamine (0·15 M)	O_2	11·7
Glucose (5×10^{-3} M)	O_2	32·4
Glucose (5×10^{-3} M) + glucosamine (0·15 M)	O_2	19·5
Adenosine ($1·7 \times 10^{-3}$ M)	O_2	32·7
Adenosine ($1·7 \times 10^{-3}$ M) + glucosamine (0·15 M)	O_2	24·0
Inosine ($1·7 \times 10^{-3}$ M)	O_2	36·0
Inosine ($1·7 \times 10^{-3}$ M) + glucosamine (0·15 M)	O_2	25·5

haemolytic effect of glucose was prevented by the addition of NaCl. However, even under these conditions, glucose at a concentration 10 times that of glucosamine did not completely prevent the inhibition of glyoxalase activity. A decrease in the reduced glutathione level of the erythrocyte was also observed after incubation with glucosamine (Table 7).

Effect of sodium arsenate

Prankerd & Altman (1954) observed that sodium arsenate had an inhibitory effect on the ^{32}P exchange of erythrocytes in the presence of adenosine or of additional glucose. In the present study, sodium arsenate was found also to inhibit the glyoxalase activity of aerated erythrocytes, both in the absence of an added substrate and in the presence of added glucose or adenosine (Table 8). Haemolysis was negligible at all sodium arsenate concentrations employed. Sodium arsenate also produced a fall in the reduced glutathione level under comparable conditions (Table 9).

Table 8. *Effect of sodium arsenate on the glyoxalase activity of aerated erythrocytes*

Conditions as in Table 2, except for the addition of supplements as indicated. Erythrocyte preparation was 3 days old. Gas phase $CO_2 + O_2$ (5:95).

Supplement	Glyoxalase activity: μl. of CO_2/60 min.	Glyoxalase activity: Difference (%)	Haemolysis (%)
None	279	—	5
Sodium arsenate ($0·16 \times 10^{-2}$ M)	277	—	3
Sodium arsenate ($0·4 \times 10^{-2}$ M)	264	− 5	4
Sodium arsenate ($1·6 \times 10^{-2}$ M)	231	−17	6
Sodium arsenate (4×10^{-2} M)	188	−33	3
Sodium arsenate ($10·8 \times 10^{-2}$ M)	123	−56	5
None	254	—	7
Sodium arsenate (8×10^{-2} M)	146	−43	4
Plasma (10%)	324	—	5
Plasma (10%) + sodium arsenate (8×10^{-2} M)	223	−31	5
Glucose (10^{-4} M)	297	—	5
Glucose (10^{-4} M) + sodium arsenate (8×10^{-2} M)	197	−34	5
Adenosine (10^{-4} M)	297	—	5
Adenosine (10^{-4} M) + sodium arsenate (8×10^{-2} M)	186	−37	4

Table 9. *Effect of sodium arsenate on the reduced glutathione (GSH) level of erythrocytes*

Conditions as in Table 1. Figures for GSH are mg./100 ml. of cell suspension in saline (1:1, v/v).

Supplement	Gas phase	GSH (mg. %)
None	N_2	28·5
Sodium arsenate ($2·7 \times 10^{-2}$ M)	N_2	10·5
None	O_2	15·0
Sodium arsenate ($2·7 \times 10^{-2}$ M)	O_2	3·0
Plasma (17%)	O_2	28·5
Plasma (17%) + sodium arsenate ($2·7 \times 10^{-2}$ M)	O_2	6·0
Glucose (5×10^{-3} M)	O_2	24·0
Glucose (5×10^{-3} M) + sodium arsenate ($2·7 \times 10^{-2}$ M)	O_2	12·0
Adenosine ($1·7 \times 10^{-3}$ M)	O_2	25·5
Adenosine ($1·7 \times 10^{-3}$ M) + sodium arsenate ($2·7 \times 10^{-2}$ M)	O_2	4·5
Inosine ($1·7 \times 10^{-3}$ M)	O_2	27·0
Inosine ($1·7 \times 10^{-3}$ M) + sodium arsenate ($2·7 \times 10^{-2}$ M)	O_2	3·0

DISCUSSION

It has been amply demonstrated that mechanisms exist in mammalian tissues which are capable of both the oxidation and reduction of glutathione (see Vennesland & Conn, 1954). The processes which lead to a reduction of oxidized glutathione are predominant *in vivo*, as indicated by the presence of glutathione largely in its reduced form (see Bhattacharya, Robson & Stewart, 1955). In the erythrocyte, the maintenance of glutathione in its reduced form appears to be dependent largely on the normal metabolic activity of the cell. Plasma, which is highly effective in maintaining the reduced glutathione level (Table 1) and the glyoxalase activity (Fig. 1) of aerated erythrocytes, appears to act largely by supplying utilizable metabolites to the cell. In addition the serum proteins may exert a stabilizing influence on the glyoxalase system.

The chief metabolite of the erythrocyte is glucose, and this substance, as first indicated by Meldrum (1932) and Jowett & Quastel (1933), is extremely active in maintaining the glyoxalase activity (Table 2) and the reduced glutathione level (Table 1) of aerated cells. An effect on the glyoxalase system is evident on the addition of glucose in concentrations well within the physiological range, suggesting that this mechanism for the maintenance of glutathione in its reduced form may be operative also *in vivo*. The glutathione reductase system, which catalyses the reduction of oxidized glutathione by reduced TPN and, to a lesser extent, by reduced DPN (Francoeur & Denstedt, 1954; Racker, 1955), is present in the erythrocyte (Meldrum & Tarr, 1934; Rall & Lehninger, 1952; Francoeur & Denstedt, 1954; Collier & McRae, 1955). The oxidation of glucose and glucose metabolites may therefore be linked with the reduction of glutathione by this system. In addition, the metabolism of glucose is required for the synthesis of glutathione (Krahl, 1953). Although glutathione can be synthesized by mature erythrocytes (Dimant, Landsberg & London, 1955; Elder & Mortensen, 1956), in the present experiments the absence of glutathione precursors and the short-term nature of most of the experiments would suggest that synthesis is not an important factor.

In addition to glucose and other sugars, adenosine and its deaminated derivative inosine are also effective in the maintenance of the glyoxalase activity (Table 3) and the reduced glutathione level (Table 1) of aerated erythrocytes. The metabolism of adenosine and inosine by the erythrocyte appears to be initiated by their phosphorylysis to form ribose phosphates (Dische, 1938, 1951). A purified nucleotide phosphorylase prepared from erythrocytes has been found to be active only with inosine or guanosine as substrate, which suggests that adenosine must first be converted into inosine (Gabrio & Huennekens, 1955). The experiments of Prankerd & Altman (1954) suggest that the ribose phosphate formed is converted into glyceraldehyde 3-phosphate, from which the depleted stores of 2:3-diphosphoglyceric acid are replenished. Rubinstein, Kashket & Denstedt (1956) have observed the accumulation of this last substance, as well as of lactic acid, on the storage of human cells with adenosine or inosine. The metabolism of adenosine leads also to the formation of fructose 1:6-diphosphate and hexose monophosphate (Dische, 1938, 1951). The subsequent oxidation of glucose 6-phosphate via the triphosphopyridine nucleotide-(TPN)-dependent glucose 6-phosphate dehydrogenase system, coupled with the reduction of oxidized glutathione (Francoeur & Denstedt, 1954; Collier & McRae, 1955), may be responsible for the observed effect on the reduced glutathione level (Table 1) and on the glyoxalase activity (Table 3).

Glucosamine was found to decrease the glyoxalase activity (Table 5) and the reduced glutathione level (Table 7) of aerated erythrocytes. Since the metabolism of glucose appears to be intimately involved in the maintenance of glutathione in its reduced form, it would seem logical to assume that this effect of glucosamine is associated with its inhibitory effect on glucose metabolism (Wick *et al.* 1955; Nakada *et al.* 1955). The production of an inhibition of glyoxalase activity in the absence of glucose, either added or endogenous, indicates that the effect of glucosamine is not necessarily a result of an inhibition of the initial phosphorylation of glucose. It is of interest in this regard that glucosamine 6-phosphate inhibits the glycolysis system of *Schistosoma mansoni* (Bueding & MacKinnon, 1955) and the glucose 6-phosphate dehydrogenase system of yeast (Glaser & Brown, 1955).

Glucose has been in general use in the preservation of erythrocytes since the observation by DeGowin, Harris & Plass (1939) that the haemolysis of stored blood was considerably delayed by the addition of this substance. Similarly, adenosine and inosine have been found in recent years to increase the survival time of stored erythrocytes (Donohue, Finch & Gabrio, 1956; Rubinstein *et al.* 1956). The mechanism by which these substances stabilize the erythrocyte during storage is not fully known. The maintenance of adenosine triphosphate levels by glucose (see Denstedt, 1953) and by adenosine or inosine (Gabrio, Finch & Huennekens, 1956), suggests that the production of adenosine triphosphate may be required for the maintenance of the intact cell structure. However, another factor may be operative. It has been suggested by a number of investigators (Fegler, 1952; Benesch & Benesch, 1954; Flanagan, Beutler, Dern & Alving, 1955; Sheets, Hamilton & DeGowin, 1956) that substances

containing sulphydryl groups, including reduced glutathione, play an important role in the maintenance of the intact erythrocyte structure. In this regard it is of interest that the increased rate of spontaneous haemolysis on the exposure of erythrocytes to oxygen (Fishman & Hunter, 1951) has been correlated with a corresponding decrease in reduced glutathione level (Fegler, 1952). The glyoxalase activity of erythrocytes stored in citrate at 5° remains normal for about 20 days and then decreases rapidly, reaching zero after 30–40 days of storage (Alivisatos, 1949). The glyoxalase activity is maintained for a much longer period when a citrate–glucose medium is employed. Since the enzyme moiety of the glyoxalase system is relatively stable on storage (Keilin & Wang, 1947), variations in reduced glutathione level are probably involved. The protective effect of glucose, adenosine and inosine on stored erythrocytes may therefore be related, in part at least, to the ability of these substances to maintain the reduced glutathione level of the cells.

SUMMARY

1. The factors affecting the oxidation and reduction of glutathione in mature mammalian erythrocytes have been investigated.

2. The glyoxalase activity of aerated erythrocytes is increased by plasma in low concentrations, by glucose, by adenosine and inosine and by bovine albumin.

3. Glucosamine and sodium arsenate, on the other hand, produce a fall in glyoxalase activity.

4. In all instances except that of albumin a corresponding alteration of reduced glutathione level was produced by the addition of each of these materials.

5. Plasma and serum albumin in high concentrations produce, under the conditions employed, a decrease in glyoxalase activity which is overcome by the further addition of methylglyoxal, suggesting that the fall in activity is due to a decrease in substrate concentration.

6. The importance of glucose, adenosine and inosine in the maintenance of the reduced glutathione level is discussed in relation to the storage of erythrocytes.

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The Electrokinetic Properties of *Aerobacter aerogenes*

A COMPARISON OF THE PROPERTIES OF NORMAL AND CRYSTAL VIOLET-TRAINED CELLS

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In a previous communication (Lowick & James, 1955) we reported marked changes in the electrophoretic behaviour of a strain of *Aerobacter aerogenes* during growth in the presence of crystal violet. The alteration of the surface, as followed electrophoretically, depended on the age of the untrained parent culture used for inoculation; further, it occurred only during actual cell division in the presence of the drug. Once a population of type C (i.e. extreme) behaviour was produced, it was stable on subsequent subculture in both the presence and the absence of the dye. In the present paper we report an investigation of this strain in an attempt to explain these altered electrokinetic properties.

It seemed reasonable that the electrokinetic properties of different strains of bacteria on treatment with suitable reagents, e.g. surface-active agents, under carefully controlled conditions of pH and ionic strength would be mainly dependent on the nature of the surface constituents. Dyar & Ordal (1946) have shown the sensitivity of bacteria of widely different taxonomic groups to treatment with both anionic and cationic surface-active agents. In general these workers found that the presence of sodium tetradecyl sulphate produced no change, or a slight or a marked increase in the electrophoretic mobility. The cationic surface-active agent, cetylpyridinium chloride, always produced the same general pattern of decrease of charge—reversal of charge and finally stabilization of charge. A 'fattened' strain of *Micrococcus aureus*, known to contain a greater amount of fatty substance than the normal strain, was more sensitive to both the anionic and cationic detergents. This was attributed to an increase in the amount of lipid material in the surface of these cells. Later Dyar (1948) described a method of characterizing cell surfaces in which chemical and enzyme treatments were employed to remove parts of the cell surface. In this way both lipid and amphoteric material were detected and removed from the surface of *M. aureus*.

Using the same technique, Douglas (1955) concluded that the absence of any effect of sodium dodecyl sulphate on *Bacillus subtilis* spores showed surface lipid to be absent, whereas with *Bacillus megatherium* spores the increase in mobility in the presence of this detergent indicated the presence of some lipid. Further evidence in support of the difference between the surfaces of these two spores was obtained from a study of the variation of their mobilities in solutions of different pH values, under conditions of constant ionic strength.

In the light of this previous work we considered that the difference of electrophoretic behaviour between the normal cells of *Aero. aerogenes* and those trained to crystal violet must be due to a change of surface constituents. Accordingly, we have investigated the effect of surface-active agents on the mobilities of these two strains. In addition, we have attempted to correlate changes of mobility on treatment with various organic solvents with the fractions extracted, as measured by ultraviolet-absorption spectra.

EXPERIMENTAL

Cultures. Aero. aerogenes was grown at 40° with aeration in a simple synthetic medium containing glucose, potassium dihydrogen phosphate, ammonium sulphate and a small quantity of magnesium sulphate (Lowick & James, 1955). The organisms were freeze-dried from aqueous suspension (rather than from serum suspension, to avoid contamination of the surface with protein) and were resuspended in the buffer solution when required. Cells stored in this way remained viable during the period of observation. This drying technique had no effect on the mobility of either the normal bacteria or those trained to crystal violet.

Cells trained to crystal violet with type C electrokinetic properties were chosen for the detailed study of altered surface characteristics for two reasons: first, these cells with very low mobility values have undergone the greatest change in the surface properties incurred by training to the dye. Secondly, cell populations of this type exhibit a homogeneous distribution of mobilities, enabling an average mobility to be determined (compare type B, Lowick & James, 1955). It is more convenient to follow changes in the average mobility of a strain produced by different chemical treatments than to detect changes in histograms which depict a heterogeneous population (type B).

Electrophoretic measurements. Suspensions for electrophoretic measurements were prepared by shaking a sample of the freeze-dried organisms with mixed phosphate buffer solution (1 part of M/150 KH_2PO_4 to 1 part of M/150 $Na_2HPO_4,12H_2O$) of ionic strength 0·013 and pH 7·00. The technique for determining the mobility and mobility

distribution of cell suspensions has already been described (Lowick & James, 1955; Moyer, 1936). The micro-electrophoresis cell was calibrated with human red-blood cells for which the mobility is 1·31 μ./sec./v/cm. in M/15 phosphate buffer solution, pH 7·35 (Abramson, 1929). Cells of *Aero. aerogenes* have an average mobility of 2·37 $\pm$ 0·04 μ./sec./v/cm. (coefficient of variation, 8%) in phosphate buffer solution of ionic strength 0·013, pH 7·00 (Lowick, Loveday & James, 1956).

RESULTS

Variation of electrophoretic mobility accompanying the first sub-culture in medium containing crystal violet

When *Aero. aerogenes* is first grown in synthetic medium containing 5·6 mg. of crystal violet/l., three different types of mobility distribution can be obtained, according to the age of the parent inoculum: for a parent of age less than 8 hr., the population at the end of the growth phase has a histogram which is typically Gaussian and similar to that for cells grown in drug-free medium (type A). A parent of age 8–36 hr. gives rise to a heterogeneous population with mobility values varying from 0·4 to 2·70 μ./sec./v/cm. (type B). A parent culture over 48 hr. old gives rise to a population of cells whose mobilities have a Gaussian distribution centred round very low mobility values (type C). Fig. 1 shows the three types of behaviour and gives the corrected absolute values for the electrophoretic mobility (Lowick *et al.* 1956). It can be seen, by comparing this diagram with Fig. 2 of our earlier paper (Lowick & James, 1955), that the new values have not affected the mobility distributions, but merely displaced the histograms towards higher values.

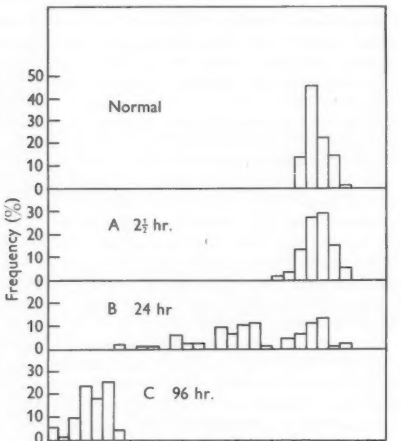

Fig. 1. Effect of the age of the inoculum of untrained cells on the mobility distribution after growth in media containing 5·6 mg. of crystal violet/l. The times 2½, 24 and 96 hr. refer to the age of inoculum; A, B and C refer to the types of behaviour.

Effect of pH *on the mobility of normal and trained cells*

The buffer solutions used in the range pH 2·8–6·9 were prepared from M/300 citric acid and M/150 disodium hydrogen phosphate adjusted to constant ionic strength (0·02) by the addition of sodium chloride. The extreme acid solution was 0·01 N hydrochloric acid, also at constant ionic strength (0·02).

The mobility–pH curves for the two strains are shown in Fig. 2. The response of the two types is considerably different; the normal cells give a curve similar to that obtained for inert surfaces, and the trained cells a curve (with an isoelectric point at pH 3·5) similar to that of some protein and lipid surfaces (Price & Lewis, 1933; Abramson, 1933).

The act of suspension of the normal cells in acid solution caused no surface damage, since when washed after this treatment and resuspended in normal phosphate buffer solution at pH 7·00 their mobility returned to the normal value. The mobility

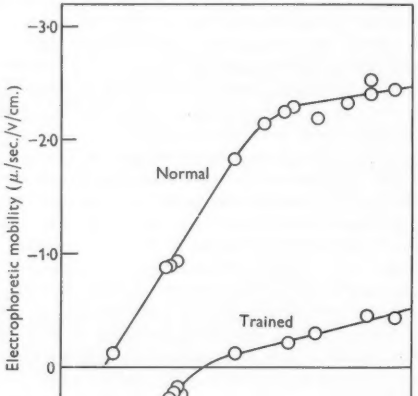

Fig. 2. Mobility–pH curves of normal and crystal violet-trained cells of *Aero. aerogenes*.

Table 1. *Effect of washing on the electrophoretic mobility of the organisms after treatment at low* pH *values*

Treatment of cells	pH of test soln.	Ionic strength	Mobility (μ./sec./v/cm.) Normal	Trained
Suspended in M/300 phosphate buffer (control)	7·00	0·013	−2·37*	−0·39
Suspended in 0·01 N-HCl	1·94	0·02	−0·12	+1·17
Suspended in 0·01 N-HCl, washed and suspended in M/300 phosphate buffer	7·00	0·013	−2·44	−0·54
Suspended in citrate–phosphate buffer (control)	6·89	0·02	−2·41	−0·31
Suspended in citrate–phosphate buffer	2·93	0·02	−0·87	+0·28
Suspended in citrate–phosphate buffer at pH 2·93, washed and suspended in citrate–phosphate buffer	6·89	0·02	−2·43	−0·43

* The sign indicates the charge carried.

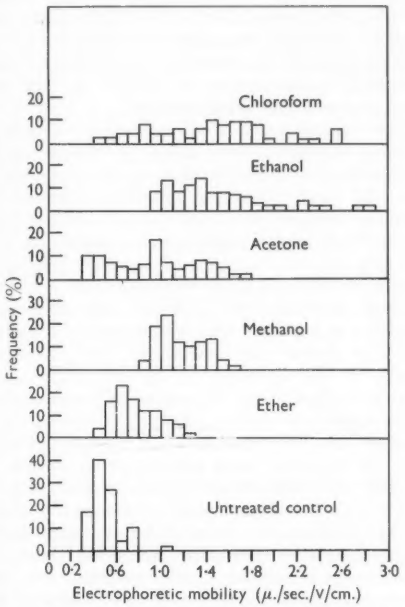

Fig. 3. Effect of solvents on the mobility distribution of trained cells of *Aero. aerogenes*.

of the trained cells, however, after similar treatment, returned to a more negative value than the control (Table 1).

Effect of organic solvents on Aerobacter aerogenes

A known weight of the dried bacteria (generally 0·01 g.) was shaken for 4 days with 40 ml. of ether, methanol, ethanol, acetone or chloroform. The organisms were removed on the centrifuge and the supernatant liquid was saved (see below). The cells were then washed twice and resuspended in the normal phosphate buffer solution.

Electrophoretic mobility. The mobility distribution of normal cells was unaltered after shaking with any of these solvents. When the same treatment was applied to trained cells, however, the histograms were greatly altered (Fig. 3). Ether and methanol caused a slight increase in the average mobilities of these cells, but the distribution ranges were not significantly increased. Acetone and ethanol produced considerable increases in mobility values and the final histograms showed a heterogeneous population. A very widespread heterogeneous population was obtained on treatment with chloroform or a mixture of chloroform and methanol, and many mobility values were in the same range as those for normal untrained cells. The final histogram obtained with any solvent was independent of the time of shaking, for periods of 2–96 hr.

Absorption spectra of the extracts. After the cells had been treated with the different solvents, the supernatant liquid was evaporated to dryness. The residue was then dissolved in absolute alcohol and the ultraviolet-absorption spectrum determined with a Unicam SP. 500 spectrophotometer. When the same weight of bacteria was shaken for different periods, ranging from 2 to 96 hr., it was observed that the absorption increased with shaking time until a maximum was attained. Extracts of both types of bacteria with ethanol, ether and acetone gave absorption spectra with similar intensities when equal amounts of bacteria were taken. Methanol was, however, a much poorer solvent.

The absorption spectra of the extracts from the two types of cells were essentially the same in the ultraviolet range (Fig. 4), consisting of an intense peak at 225–230 mμ., presumably due to the presence of lipid-like material (Miller, Brown & Burr, 1938), and a lesser peak at 250–252 mμ. due to the presence of purines or pyrimidines (possibly nucleic acids). A comparison of the relative intensities of these absorption spectra shows that the trained cells contain approximately the same amount of these materials as an equal weight of normal cells. The trained cells are considerably

smaller than the normal cells; 1 mg. of normal cells contains $32{\cdot}5 \times 10^8$ organisms, whereas 1 mg. of trained cells contains $81{\cdot}5 \times 10^8$ organisms.

Crystal violet is soluble in all the solvents used (except ether) and was therefore extracted by this shaking procedure. This was shown by the additional peak in the absorption spectra of the extract of trained cells at 580 mμ. due to the dye. From the intensity of this peak the amount of crystal violet extracted from 1 mg. of bacteria was calculated as being 470 μg. After such extraction the bacteria were colourless.

Although the solvents have generally removed the dye from the trained organism, their mobility distribution never returns to that characteristic of untrained cells.

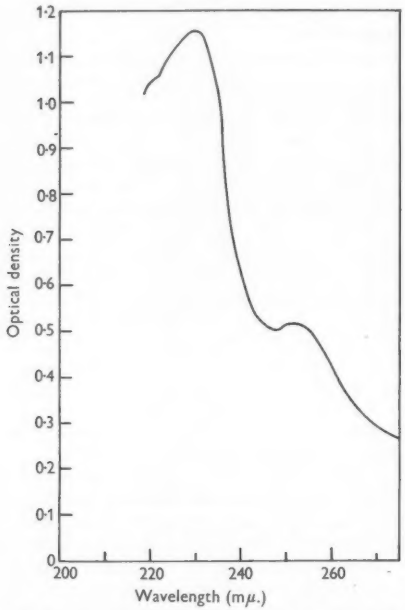

Fig. 4. Absorption spectrum in absolute ethanol of the ether extract of normal cells of *Aero. aerogenes*. 15 mg. of bacteria was shaken for 4 days with 30 ml. of ether, the solvent was evaporated and the residue dissolved in 200 ml. of absolute ethanol.

Effect of surface-active agents on the mobility of normal and trained cells

To characterize the surface of normal and trained bacteria their mobilities were determined in buffer solution containing a surface-active agent. Cetylpyridinium chloride was chosen as the cationic and sodium dodecyl sulphate as the anionic surface-active agent. Concentrations up to 0·02 M of each of these were prepared in buffer solution and mixed with the bacterial suspension in buffer solution to give the appropriate concentration of the detergent. The final concentration of bacteria in each suspension was 3×10^8 organisms/ml.

The normal cells were insensitive to sodium dodecyl sulphate, there being no change in the electrophoretic mobility at concentrations up to mM. In cetylpyridinium chloride, however, the mobility became less negative at a concentration of about 50 μM, and at higher concentrations the cells actually possessed a positive charge. The cells of the trained strain were much more sensitive to sodium dodecyl sulphate, the mobility becoming more negative at 0·1 mM. At a concentration of mM the mobility showed an increase of 800% over the value in the absence of surface-active agent (Table 2). In addition the trained cells were slightly more sensitive to cetylpyridinium chloride than the normal cells, the curve crossing the concentration axis at a much lower concentration (60 μM for trained compared with 600 μM for normal) (Fig. 5).

The physical effect of these surface-active agents on the surface was investigated by washing the organisms, after treatment with the detergent, twice with phosphate buffer solution and then determining their mobility in this medium. The results (Table 3) show that this washing does not

Table 2. *Effect of surface-active agents on the electrophoretic mobility of* Aerobacter aerogenes

Strain and treatment	Surface-active agent	Electrophoretic mobility (μ./sec./v/cm.) Control	Electrophoretic mobility (μ./sec./v/cm.) In mM	Change from control (%)	Concn. at which departure from control occurs
Normal	SDS†	−2·35	−2·39	2	Above mM
Trained	SDS	−0·46	−4·18	810	0·1 mM
Normal treated with ether	SDS	−2·31	−2·52	9	0·5 mM
Trained treated with ether	SDS	−0·88	−5·50	525	0·1 mM
Trained treated with $CHCl_3$ + methanol	SDS	−2·32*	−4·18	80	0·1 mM
Normal	CPC	−2·36	+0·80	134	50 μM
Trained	CPC	−0·48	+0·89	286	10 μM

* Population heterogeneous.

† In this and subsequent tables SDS represents sodium dodecyl sulphate and CPC represents cetylpyridinium chloride.

Table 3. *Effect of washing on the electrophoretic mobility of organisms after treatment with surface-active agents*

Treatment of the cells	Electrophoretic mobility (μ./sec./v/cm.) Normal	Trained
Suspended in M/300 phosphate (control)	−2·48	−0·39
Suspended in buffer + mM SDS	—*	−3·8
Suspended in buffer + mM SDS, washed and suspended in M/300 phosphate	—*	−4·10
Suspended in buffer + mM CPC	+0·35	+1·23
Suspended in buffer + mM CPC, washed and suspended in M/300 phosphate	−1·90	−0·66

* Not tested owing to the lack of effect of SDS on normal cells.

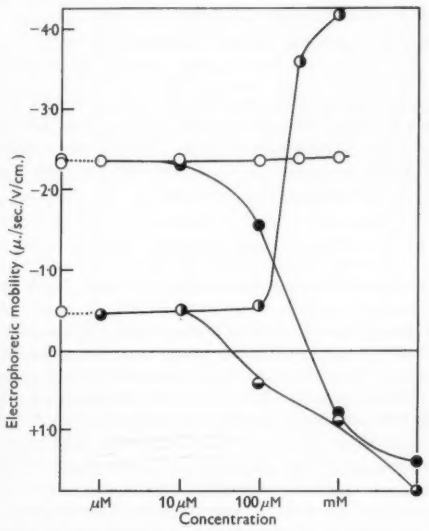

Fig. 5. Electrophoretic mobility of normal and trained cells of *Aero. aerogenes* in the presence of surface-active agents. ○, Normal cells in SDS; ●, normal cells in CPC; ◑, trained cells in SDS; ◒, trained cells in CPC.

completely remove the cetylpyridinium chloride from the surface of normal cells. The effect of sodium dodecyl sulphate on the trained cells is irreversible, possibly due to the formation of a stable lipid–detergent complex. Treatment of the trained cells with cetylpyridinium chloride followed by washing results in an increased negative mobility value over the control.

The effect of sodium dodecyl sulphate was tested on bacteria which had been treated with ether for 4 days. Normal cells were now slightly more sensitive, and the trained cells were less sensitive, to this reagent, but still showed a considerable increase in their mobility with mM sodium dodecyl sulphate (Table 2). Trained bacteria treated with other solvents gave similarly high mobility values in the presence of mM sodium dodecyl sulphate. The distributions which were widespread in the controls (e.g. chloroform–methanol) became homogeneous in the presence of the surface-active agent.

The differences in behaviour of normal and trained strains towards surface-active agents can most probably be attributed to a higher content of lipid-like material in the surface of the trained organisms. This material is partly extracted by the ether.

Effect of lipase on the surface properties of normal and trained cells

A sample of each of the freeze-dried strains (0·01 g.) suspended in 25 ml. of phosphate buffer solution at pH 7·00 was treated with lipase (Solmedia Ltd.; final concentration, 1 % w/v) at 40° for about 18 hr. Controls, in which the enzyme had previously been inactivated by heat treatment, were always included. The suspension was centrifuged and the organisms were washed twice with phosphate buffer solution and finally resuspended in that menstruum. The mobility distribution of each suspension was determined in the presence and absence of mM sodium dodecyl sulphate.

The average values (Table 4) show that the active lipase is without effect on the electrophoretic mobility of the normal cells in the presence and absence of sodium dodecyl sulphate. In contrast, lipase brings about a marked increase in the mobility of the trained cells to a value characteristic of normal untrained and untreated cells. Further, these cells are no longer sensitive to the presence of sodium dodecyl sulphate. Inactivated lipase is without effect on the mobility of normal cells. It increases the average mobility of trained cells by some 40 % and reduces their sensitivity to sodium dodecyl sulphate by approximately the same amount. These changes are, however, negligible when compared with those resulting from the use of the active enzyme.

Table 4. *Effect of lipase on the electrophoretic mobility of normal and trained cells of* Aerobacter aerogenes

	Electrophoretic mobility (μ./sec./v/cm.)	
	Normal cells	Trained cells
Untreated cells (controls)		
Suspended in buffer solution	2·40	0·45
Suspended in buffer solution + mM SDS	2·39	4·44
Percentage increase in the presence of SDS	0	890
Cells treated with active lipase		
Suspended in buffer solution	2·45	2·31
Suspended in buffer solution + mM SDS	2·29	2·34
Percentage increase in the presence of SDS	−6*	0
Cells treated with inactive lipase		
Suspended in buffer solution	2·43	0·63
Suspended in buffer solution + mM SDS	2·31	4·00
Percentage increase in the presence of SDS	−5*	540

* These values have little significance, as they are only just outside the limits of experimental error.

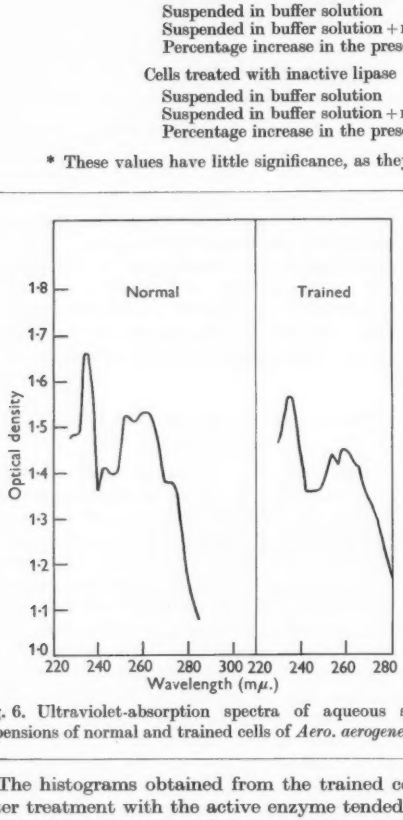

Fig. 6. Ultraviolet-absorption spectra of aqueous suspensions of normal and trained cells of *Aero. aerogenes*.

The histograms obtained from the trained cells after treatment with the active enzyme tended to be bimodal in form, there being a small percentage (about 10 %) of the population with the original low mobility value. A similar enzymic treatment of killed cells (2 hr. at 55°) with active lipase produced the same result as for viable cells.

Absorption spectra of aqueous suspensions of normal and trained cells

The ultraviolet-absorption spectra of cells of the normal and trained strains were determined in aqueous suspension by the method of Shibata, Benson & Calvin (1954) (Fig. 6). The absorption spectra are similar, maxima occurring at 235, 254 and 260 mμ. The maxima at 260 mμ. may be due to deoxypentosenucleic acid. The other maxima probably correspond to those observed on the solvent extracts of the bacteria.

DISCUSSION

The electrophoretic behaviour of cells of *Aerobacter aerogenes* trained to crystal violet is markedly different from that of untrained cells. It is evident from the colour of the trained organisms that the dye has been absorbed. The amount absorbed on the surface from the low dye concentration in the growth medium is insufficient to produce the change in mobility (Lowick & James, 1955). The dye is not removed by washing with phosphate buffer solution, and the mobility is unchanged by this treatment (Lowick & James, 1955). The differences in the electrokinetic properties are due to an alteration in the nature of the cell surface and not to the effect of the dye combined with surface components of the trained organism. This is supported by two independent observations: First, repeated and prolonged growth of the trained cells (type C behaviour) in the absence of the dye caused no alteration in the mobility distribution (Lowick & James, 1955). The large increase of surface area which occurred during growth in the absence of the dye precludes the possibility of any significant amount of crystal violet remaining on the surface. The organisms were colourless and no dye was ever detected by examination of ethanolic extracts of the organisms. Secondly, organic solvents (except ether) extract the dye from the trained organisms, but the mobility distribution, although in some cases undergoing a change, never returns to the value for normal untrained cells. A further type of training to crystal violet-type A (reported pre-

viously) is worth recalling. Cells of this training series, although containing considerable amounts of the dye, possessed a mobility distribution which was indistinguishable from that of untrained cells.

Thus it can be concluded that the outermost surface layers of normal and trained cells (type C) are of different chemical or physical structure, or both. This fact is amply illustrated by the large quantitative differences observed in the effect of pH and surface-active agents on the mobility values (Figs. 2, 5). Some idea of the nature of these two cell surfaces can be obtained by comparing these results with those for model surfaces, e.g. polysaccharide, protein and lipoprotein, obtained by previous workers.

For the cells of *Aero. aerogenes* trained to crystal violet, the form of the mobility–pH curve (with an effective isoelectric point at pH 3·5) is similar to those of protein (Abramson, 1933) and lecithin (Price & Lewis, 1933) surfaces—ionogenic, amphoteric—and to those of some sterols (Moyer, 1934). In addition these cells show a marked increase in mobility in the presence of the anionic surface-active agent sodium dodecyl sulphate. Droplets of a hydrocarbon and of lipids acquire a greater negative charge in the presence of these surface-active agents, a phenomenon attributed to the solution of the hydrocarbon ends of the detergent molecules in their surface with the negatively charged polar groups oriented outwards (Dyar, 1948). Thus the material in the cell surface responsible for the increase in mobility is most probably lipid. Treatment of these trained cells with organic solvents, particularly ether, makes them slightly less sensitive to sodium dodecyl sulphate. This may be due to the removal of some of the fatty lipid material from the surface, resulting in a surface in which less of the sodium dodecyl sulphate can dissolve.

In contrast the mobility–pH curve for the untrained cells, which is essentially independent of pH above pH 5·0, is quite unlike those for protein or lipid surfaces. Rather does it resemble in form the curve for an inert surface (Abramson, Moyer & Gorin, 1942), and those for the smooth forms of bacteria which almost certainly have a polysaccharide surface. The anionic surface-active agent has no effect on the mobility [cf. cellulose and glass particles (Dyar, 1948)], but the cationic agent (cetylpyridinium chloride) results in neutralization and reversal of charge, as would be expected.

Further evidence about the nature of the surfaces was obtained from a study of the effect of lipase on the cells. This enzyme has a pronounced effect on the mobility of trained cells. After treatment, these cells had a mobility characteristic of untrained cells; further, like untrained cells, they were insensitive to sodium dodecyl sulphate. In contrast, lipase had no effect on the mobility of normal bacteria either in phosphate buffer suspension or in the presence of sodium dodecyl sulphate, suggesting the absence of lipid in the surface of these cells.

Bacterial cell walls, particularly those of the Gram-negative bacteria, are known to contain a considerable amount of lipid material (Salton, 1956). The ultraviolet-absorption spectra of the normal and trained organisms and the solvent extracts are consistent with this observation. The spectra show that both strains contain approximately the same amounts (w/w) of the different materials, but as the trained are of approximately one-third the size of the normal cells it would appear that there would be less material (lipid, nucleic acids, etc.) per cell. This lipid in the trained cells is exposed and is readily detected. This is in contrast to that in the normal cell, where the lipid probably constitutes the deeper layers in the cell wall. Treatment of normal cells with solvents does not affect their mobility, but renders them slightly more sensitive to sodium dodecyl sulphate, indicating the removal of an outer layer, or layers, and the consequent laying bare of (at least) a portion of the lipid material.

We believe, from the evidence reported, that cells of *Aero. aerogenes* which have been trained to grow in the presence of crystal violet contain lipid material exposed on the surface, whereas the surface of the normal cells is predominantly polysaccharide. Further work on the effect of specific enzymes on the electrical properties of these surfaces is in progress.

SUMMARY

1. The mobility–pH curve of the normal strain of *Aerobacter aerogenes* is similar to that of a polysaccharide surface; that of the trained strain is similar to the curve obtained for protein surfaces.

2. The greater sensitivity of the trained cells towards both anionic and cationic surface-active agents suggests that a greater amount of lipid material is present in their surface.

3. Treatment with organic solvents does not affect the mobility distribution of normal cells, but histograms obtained from the trained cells were altered to varying degrees. Ultraviolet-absorption spectra of the extracts from normal and trained cells were similar.

4. Extraction with solvents renders the normal cells slightly more sensitive and the trained cells less sensitive to sodium dodecyl sulphate.

5. Lipase has no effect on the mobility of normal cells, but increases the value for trained cells to that of a normal untrained strain. The trained cells, so treated, are insensitive to sodium dodecyl sulphate.

6. The experimental observations, discussed in the light of model surfaces of known structure, are consistent with the hypothesis that the cells of *Aero. aerogenes* trained to crystal violet have a surface which is lipid in nature, in contrast to that of the normal cells, which is polysaccharide.

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The Metabolism in the Rat of Naturally Occurring (+)-14-Methylhexadecanoic Acid

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The recent isolation of branched-chain fatty acids from animal fats (Hansen, Shorland & Cooke, 1952) has aroused interest in the fate of such acids as dietary components. Hitherto, feeding experiments with branched-chain acids were carried out mainly in relation to the effects of such acids present in synthetic fat. Thus Appel, Böhm, Keil & Schiller (1947) fed glycerides of the higher branched-chain fatty acids with methyl, ethyl or propyl side chains to goats, whereas Weitzel (1951) fed dogs with α-substituted myristic and stearic acid triglycerides. The metabolism of the acids was followed by determining the nature of the ether-soluble breakdown products in the urine. In addition, without giving experimental evidence, Appel *et al.* (1947) stated that 13·6 % of the branched-chain acids fed in synthetic fat to goats was found in the depot fats. Weitzel (1951) estimated that 90 % of the α-substituted myristic and stearic acids fed to dogs was metabolized, but he did not determine the storage of these acids in the body.

In the present study the branched-chain acid (+)-14-methylhexadecanoic acid, naturally occurring up to approximately 0·5 % in ruminant fat, has been fed to rats to determine its storage, excretion, breakdown and effect on growth.

Because of the difficulty of isolation of traces of branched-chain fatty acids from animal fats, metabolic studies without the use of isotopically labelled compounds are not normally feasible. However, as the acid used in this work is optically active it has been possible to utilize this property as a sensitive method of estimation.

EXPERIMENTAL

Rats of the Wistar strain maintained as an inbred colony for the past 22 years were used. They were fed on the experimental diet from weaning (40–50 g.) until they reached 160 g.

The 'fat-free' basal diet used was similar to that described by Longenecker (1939). It consisted of (%): casein, 20; corn starch, 60; sucrose, 9; salt mixture, 4; agar-agar, 2; dried yeast, 5. The diet was computed to contain not more than 1·36 % of fat.

The animals were divided into groups, each with two subgroups consisting of five bucks and five does. Group A (controls) were given the basal 'fat-free' diet; group B received the same diet but with a supplement of 0·1 g. of C_{17} *anteiso*-acid (from a preparation obtained by Hansen *et al.* 1952)/rat/week. Altogether seven such supplements were given, after which the bucks returned to the basal diet for 3 weeks, and the does for 7 weeks. The animals were then killed. The faeces of bucks and does were collected for 48 hr.

after each dose. By the use of a jet of air they were freed from hairs and food particles, and were then stored in alcohol at 0°, and combined, giving a total weight of 54·8 g. of dry matter.

In the second week of the experiment, it was found that owing to scattering of the 'fat-free' food, the weights began to decline. To prevent this complication, the 24 hr. supply of food was made into a paste. The average time required to attain the target weight of 160 g. was taken as an indication of the growth rate. The group A bucks required 9½ weeks and the group B bucks 10 weeks. The does in both groups required over 14 weeks.

At the conclusion of the feeding period the animals were decapitated and the blood was drained out. The alimentary canal was discarded and the bodies were minced and then boiled for 20 min. in 2 vol. of 95% ethanol. After distilling off the alcohol *in vacuo* the tissues were exhaustively extracted with boiling acetone, and then with 95% ethanol followed by peroxide-free ether. The extracts were combined and the solvents evaporated. The fat was taken up in light petroleum (b.p. 50–60°), filtered, and the solvent removed at 100° *in vacuo*. The faecal lipids were prepared in the same way.

The fats were saponified and the unsaponifiable matter was extracted as described by Hilditch (1947). The soaps were acidified and the fatty acids crystallized five times from 10 vol. of acetone at −33°. The liquid, high-iodine value fractions were bulked and converted into methyl esters. Previous experience (Hansen, Shorland & Cooke, 1954) has shown that the *anteiso*-acids occur in these fractions when this method of crystallization is used. The methyl esters were fractionally distilled at 0·1 mm. in a spiral fractionating column based on column E (Shorland, 1952). The optical activity of the fractions was determined and the proportions of (+)-14-methylhexadecanoic and (+)-12-methyltetradecanoic acids were calculated from the values given by Weitkamp (1945) for the pure acids. No optical activity was observed apart from that shown by fractions corresponding to methyl esters of C_{15} and C_{17} acids (see Tables 1 and 2). No activity was found in the fractions from the control group A.

The amount of buck fat fractionated represents 75·0 g. out of a total of 110·0 g. The total fat, therefore, contained 0·003 g. of C_{15} *anteiso*- and 0·33 g. of C_{17} *anteiso*-acids.

Out of a total of 91·3 g. of doe fat 76·2 g. was used, so that the total C_{15} *anteiso*-acid present was 0·014 g., whereas the C_{17} *anteiso*-acid amounted to 0·28 g.

Since insufficient group B combined faecal fat was available for fractionation, 20·42 g. of fat from group A does, devoid of optical activity, and 0·95 g. of group B faecal fat were combined. After removal of unsaponifiable matter as described by Hilditch (1947) the fatty acids were crystallized as outlined above. The liquid fractions were combined and subjected to ester fractionation, with column E (Shorland, 1952) at 0·1 mm., and 0·16 g. of optically active acid was obtained (Table 3). The C_{17} *anteiso*-acid present in the total faecal fat (3·89 g.) was therefore 0·66 g.

Table 1. *Fractional distillation of the methyl esters of the acetone-soluble fatty acids of the bucks of group B*

Fraction	Wt. (g.)	Saponification equiv.	Iodine value	α_D^{18}*	*anteiso*-Acid† (g.)
1	0·20	237·5	18·0	+0·00°	Nil
2	0·17	252·7	14·6	0·06	0·002 (C_{15})
3	6·19	267·1	84·0	0·00	Nil
4	1·89	270·8	79·4	0·10	0·038 } (C_{17})
5	4·99	293·5	83·5	0·19	0·189 }
6	9·09	296·4	96·7	0·00	Nil
7	13·33	296·6	87·2	0·00	Nil
8	2·41	297·6	85·0	0·00	Nil
Residue	5·24	353·1	95·3	—‡	—
Total	43·51				

* Rotation measured in a 1 dm. cell without dilution.
† Calculated from $[\alpha]_D^{18}$ C_{15} *anteiso* = +4·7° and $[\alpha]_D^{18}$ C_{17} *anteiso* = +5·0° (Weitkamp, 1945).
‡ Rotation not measurable because of dark colour.

Table 2. *Fractional distillation of the methyl esters of the acetone-soluble fatty acids of the does of group B*

Fraction	Wt. (g.)	Saponification equiv.	Iodine value	α_D^{18}*	*anteiso*-Acid† (g.)
1	0·61	241·7	25·6	+0·00°	Nil
2	0·85	257·4	54·3	0·07	0·012 (C_{15})
3	7·60	269·2	92·4	0·00	Nil
4	0·92	272·1	64·8	0·00	Nil
5	0·85	290·2	71·8	0·24	0·041 }
6	1·59	292·9	88·8	0·16	0·051 } (C_{17})
7	3·72	295·1	90·4	0·14	0·104 }
8	7·01	296·8	87·4	0·03	0·042 }
9	8·38	296·9	87·9	0·00	Nil
10	7·23	294·9	86·2	0·00	Nil
Residue	5·07	327·2	105·1	—‡	—
Total	42·83				

* † ‡ See Table 1.

Table 3. *Fractional distillation of the methyl esters of the acetone-soluble fatty acids of the combined faeces of group B*

Fraction	Wt. (g.)	Saponification equiv.	α_D^{18}*	C_{17} *anteiso*-Acid† (g.)
1	1·56	263·1	+0·00°	Nil
2	1·38	266·4	0·00	Nil
3	1·61	283·6	0·11	0·03
4	2·56	291·7	0·22	0·11
5	1·61	296·7	0·06	0·02
Residue	4·09	313·7	0·00	Nil
Non-sap.	0·90	—	—‡	—
Total	13·71			

* † ‡ See Table 1.

Table 4. *Metabolism of* C_{17} anteiso-*acid in the rat*

	Bucks		Does	
C_{17} *anteiso* [(+)-14-Methyl-hexadecanoic]-acid	Wt. (g.)	%	Wt. (g.)	%
Found in total fat	0·28	8·0	0·34	9·7
Found in faecal fat*	0·33	9·4	0·33	9·4
Metabolized†	2·89	82·6	2·83	80·9
Total administered	3·50	100·0	3·50	100·0
Approximate amount of C_{15} *anteiso*-acid found	0·003	0·09	0·014	0·40

* It has been assumed that equal weights of acid were present in the buck and doe faeces.
† By difference.

DISCUSSION

It will be seen from Table 4 that some 8–10 % of the dietary branched-chain acid was stored in the depot fat and that a similar amount was excreted, whereas the remainder, comprising approximately 80 %, was metabolized. It should be borne in mind, however, that the animals were not killed immediately after the last dose was administered. Although the period involved was 7 weeks for the does and 3 weeks for the bucks, the amount of (+)-*anteiso*-acids remaining in the depot fats in each case was practically identical, which suggests that the mobilization of these acids from the depots is very slow, or else that there is a large difference in the storage of these acids in does and bucks. The latter possibility, however, appears less likely. In addition, a small amount of the C_{17} *anteiso*-acid (0·1–0·4 %) was broken down to (+)-12-methyltetradecanoic acid. The presence of this latter substance is consistent with the operation of a Lynen (1954) fatty acid cycle, in which the branched-chain acids are degraded or synthesized by the successive removal or addition of acetate units.

As there were no differences in growth rates of the experimental and control groups it appears that (+)-14-methylhexadecanoic acid does not influence growth at the level used and behaves similarly to saturated *n*-fatty acids.

SUMMARY

1. Rats were fed on a 'fat-free' diet and a similar diet augmented by (+)-14-methylhexadecanoic acid at the rate of 0·1 g./rat/week, seven supplements being given.

2. It was found that 8–10 % of the acid was stored, about 10 % was excreted and approximately 80 % was metabolized. Furthermore, the presence of traces of the C_{15} *anteiso*-acid in the experimental animals, but not in the control animals, affords evidence for degradation of the C_{17} *anteiso*-acid included in the diet.

3. The addition of (+)-14-methylhexadecanoic acid to the 'fat-free' diet at the rate of 0·1 g./rat/week was without influence on the growth rate of the rat.

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The Stereoisomers of αε-Diaminopimelic Acid

2. THEIR DISTRIBUTION IN THE BACTERIAL ORDER ACTINOMYCETALES AND IN CERTAIN EUBACTERIALES*

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The occurrence in various Eubacteriales of two of the three stereoisomers of αε-diaminopimelic acid (1:5-diaminopentane-1:5-dicarboxylic acid) has been reported in a preliminary survey (Hoare & Work, 1955*a*, *b*). The most common form was the *meso* isomer, but the LL isomer occurred in *Clostridium perfringens* (syn. *Cl. welchii*) and the two propionibacteria examined. In an earlier study of the Actinomycetales (carried out before reference isomers of diaminopimelic acid were available), the nocardia were found to resemble *Mycobacterium tuberculosis* in their diaminopimelic acid content; streptomyces and an actinomyces contained what appeared, at the time, to be another amino acid differing slightly from diaminopimelic acid in its behaviour towards chromatography and enzymes (Work, 1953*a*, *b*; Work & Dewey, 1953*a*, *b*). This amino acid (then referred to as 'A') was isolated and tentatively identified as a higher homologue of diaminopimelic acid, but has now been shown to be LL-diaminopimelic acid.

This paper describes the isolation and identification of LL-diaminopimelic acid from hydrolysed streptomyces and also the distribution of the three stereoisomers among representative members of the order Actinomycetales. In view of the finding of the less common LL isomer in certain propionibacteria and clostridia, further examinations are reported of the diaminopimelic acid isomer contents of related organisms.

The following paper includes an examination of the distribution of two bacterial enzymes known to metabolize diaminopimelic acid, and compares the enzyme and diaminopimelic acid isomer contents of various organisms (Antia, Hoare & Work, 1957). A preliminary report has been given of these findings (Hoare & Work, 1956).

MATERIALS AND METHODS

Diaminopimelic acid. Reference isomers were prepared by enzymic resolution of synthetic diaminopimelic acid (Work, Birnbaum, Winitz & Greenstein, 1955). The *meso* and LL isomers were also prepared by fractional crystallization of the isomer mixture obtained from the culture filtrate of the lysine auxotroph of *Escherichia coli* 26–26 (Hoare & Work 1955*b*). Crude LL-diaminopimelic acid (amino acid 'A') was prepared from hydrolysed *Streptomyces* MA by the method used for the isolation of *meso*diaminopimelic acid from *Corynebacterium diphtheriae* (Work, 1951).

Bacteria. Organisms were grown as described in the succeeding paper, unless otherwise stated (Antia *et al.* 1957), or were used as dried cell preparations kindly presented by various workers. Hydrolysates of bacteria were prepared containing the equivalent of 83 mg. of dried cell/ml.; ethanol extracts were made from dried bacteria (Work & Dewey, 1953*a*). Cytoplasmic fractions from *Esch. coli* were prepared from the lyophilized cytoplasmic contents (580 mg.) obtained after removal of cell walls (this was kindly provided by Dr M. R. J. Salton). The preparation was dissolved by overnight stirring at +2° in 0·1 M bicarbonate buffer, pH 8·2 (25 ml.), containing 0·6 M-KCl. Insoluble debris was removed by centrifuging at 600 *g* and the supernatant solution was diluted with water (100 ml.). Successive centrifugings at 1300, 2700 and 105 000 *g* produced sediments, none being produced at 6600 *g*. The sediments and final supernatant solution were analysed for protein, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) by the method of Schneider (1945). Each fraction was also hydrolysed and examined by paper chromatography for diaminopimelic acid.

Paper chromatography. Two-dimensional chromatograms were made on Whatman no. 4 paper, with phenol (NH_3 atmosphere) and butanol–acetic acid as solvents (Work & Dewey, 1953*a*). When crude hydrolysates were examined for diaminopimelic acid, overlap with cystine was prevented by pretreating the solution with H_2O_2 (Dent, 1948). One-dimensional chromatography was carried out on Whatman no. 1 paper, by overnight irrigation with methanol–water–10 N-HCl–pyridine (80:17·5:2·5:10, by vol.), the spots being developed by dipping the sheets in ninhydrin in acetone (0·1 %, w/v) and heating at 100° for 2 min. (Rhuland, Work, Denman & Hoare, 1955; Hoare & Work, 1955*b*). The characteristic green diaminopimelic acid spots, which faded to a permanent yellow colour, were easily distinguished from the faster-fading purple spots given by other amino acids, the majority of which had higher R_F values than diaminopimelic acid. Lysine and cadaverine spots were well separated from diaminopimelic acid.

The one-dimensional system was used to separate LL-diaminopimelic acid from the *meso* and DD isomers. Crude hydrolysates were not usually suitable for direct examination unless diaminopimelic acid was present in sufficiently high concentration to be apparent as strong spots after chromatography of 20 μl. of hydrolysate. When larger amounts of hydrolysate had to be used, the diaminopimelic

* Part 1: Hoare & Work (1955*b*).

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acid spots were obscured or displaced by streaking of other amino acids; this could be partly eliminated by treating the hydrolysate with a small amount of H_2O_2 before running the chromatogram. The neutral amino acid fractions prepared by electrodialysis or paper electrophoresis of hydrolysates (Work & Dewey, 1953*a*; Hoare & Work, 1955*b*) were more generally used without H_2O_2 treatment, as the next slowest moving amino acid was well separated from diaminopimelic acid; the equivalent of 30–40 μl. of hydrolysate could then be used if necessary. Neutral fractions could not be used in greater amounts without further purification, effected by HCl fractionation on a column of Zeo-Karb 225 (Work & Denman, 1953).

Enzymic tests for isomers. One-dimensional chromatography did not differentiate between *meso*- and DD-diaminopimelic acid; these isomers were identified enzymically by means of diaminopimelic acid decarboxylase and L-amino acid oxidase, both of which attack *meso*- but not DD-diaminopimelic acid (Hoare & Work, 1955*b*; Work, 1955). Neither enzyme attacked diaminopimelic acid in untreated bacterial hydrolysates, but both were satisfactory when used on neutral amino acid fractions. Crude diaminopimelic acid decarboxylase was provided by any acetone-dried cell preparations of *Aerobacter aerogenes* or *Esch. coli* which had been grown on glucose–salt medium and which had a Q_{CO_2} (μl. of CO_2/mg. dry wt./hr.) greater than 3·0. These preparations converted both LL- and *meso*-diaminopimelic acid into lysine. The cells (10 mg.) were suspended in 0·25 M phosphate buffer, pH 7·0 (0·3 ml.), and shaken overnight at 37° with the neutral amino acid fraction under test (0·3 ml.). Samples (0·2 ml.) taken at the beginning and end of incubation were deproteinized in ethanol (0·4 ml.) and the supernatant solutions (0·1–0·2 ml.) were examined by one-dimensional paper chromatography. Suitable controls were included, of known isomers incubated with enzyme, either alone or plus test solution. The absence of DD isomer was proved when the strong '*meso*' diaminopimelic acid spot, present in the zero-time sample of the test solution, was not found in the incubated sample. If diaminopimelic acid persisted after incubation, the presence of some DD isomer was suggested. When both DD and *meso* isomers were present, incubation produced a decrease in concentration of diaminopimelic acid, accompanied by formation of lysine (shown by two-dimensional chromatography of another portion of incubate). In any case, when DD-diaminopimelic acid appeared to be present, the test was repeated with L-amino acid oxidase from *Neurospora*; 3 mg. of enzyme (activity Q_{O_2} (μl. of O_2/mg./hr.) = 10) in 0·1 M borate buffer, pH 8·6 (0·5 ml.), was incubated for 6 hr. in O_2 atmosphere with the test solution (0·3 ml.).

Partially purified diaminopimelic acid decarboxylase from *Aero. aerogenes* (Hoare & Work, 1955*b*) was used in a few cases. It was relatively specific for *meso*diaminopimelic acid and had little or no action on the LL isomer.

Optical rotations. These were measured on 5% (w/v) solutions in 5 N-HCl in 20 cm. tubes at 24°.

Chinard reaction. This was carried out as described by Chinard (1952).

RESULTS

Isolation and identification of LL-*diaminopimelic acid from* Streptomyces

Streptomyces MA (300 g. dry wt.) was hydrolysed and processed as in the preparation of *meso*-diaminopimelic acid. The neutral fraction (containing crude amino acid 'A') obtained by electrodialysis was contaminated with small amounts of other neutral amino acids. These were removed by twice precipitating with ethanol the barium salt of 'A' (Jones & Moeller, 1928), but considerable amounts of 'A' remained in solution. 'A' was later purified by ion exchange on Zeo-Karb 225 (Work & Denman, 1953). Repeated precipitation of 'A' as the hydrochloride from aqueous acetone gave 300 mg. of pure material. It had $[\alpha]_D + 36{\cdot}2$. (Found: C, 37·3; H, 6·7; N, 12·4; Cl, 15·2. Diaminopimelic acid monohydrochloride, $C_7H_{15}O_4N_2Cl$, requires C, 37·1; H, 6·6; N, 12·3, Cl, 15·7. $[\alpha]_D$ for LL isomer = + 38·5.)

Amino acid 'A' resembled authentic LL-diaminopimelic acid in all respects. Thus it behaved identically on paper chromatography in all solvent systems which separated the isomers (Rhuland *et al.* 1955), and gave the characteristic ninhydrin colour reaction on paper. On reaction with ninhydrin in acid solution (Chinard, 1952),¶ the products from 'A' and synthetic diaminopimelic acid gave identical absorption spectra. The free amino acid forms of both 'A' and LL-diaminopimelic acid were very soluble in water, crystallized with one molecule of water of crystallization (10%, w/v) and formed gels, rather than crystals, on treatment of aqueous solutions with ethanol (Work *et al.* 1955). 'A' and LL-diaminopimelic acid were only slightly decarboxylated by partially purified preparations of diaminopimelic acid decarboxylase, the extent of decarboxylation depending on the particular enzyme preparations used (Hoare & Work, 1955*b*). Crude decarboxylase preparations from *Aero. aerogenes* completely decarboxylated both samples at identical rates.

Diaminopimelic acid isomers in the Actinomycetales

The results of this examination are shown in Table 1. Six species of *Mycobacterium* all contained *meso*diaminopimelic acid in sufficient amounts to be easily identifiable when 10 μl. of the whole hydrolysate was examined (see Rhuland *et al.* 1955, Fig. 3). The neutral fractions showed no trace of the LL isomer and after testing with crude or purified decarboxylase were free from residual diaminopimelic acid, proving the absence of the DD isomer. Seven different species of *Nocardia* all resembled the mycobacteria in their high content of *meso*diaminopimelic acid, but many of them contained traces of the LL isomer (Fig. 1, spots A and B). When the decarboxylation of the neutral amino acid fractions of certain nocardia was carried out with partially purified decarboxylase, LL-diaminopimelic acid remained, but the use of crude enzyme resulted in complete decarboxylation of all the diaminopimelic acid. The LL isomer in some species of *Nocardia* was

Table 1. *Distribution of diaminopimelic acid (DAP) isomers among certain bacteria*

Bacterial hydrolysate (10 μl. = 0·8 mg. dry wt.) and neutral fraction from hydrolysate were examined by paper chromatography on Whatman no. 1 paper in methanol–water–10 N-HCl–pyridine (80:17·5:2·5:10, by vol.). Diaminopimelic acid was identified by position and by colour of spots (olive-green, fading to permanent yellow) on dipping in ninhydrin in acetone (0·1 %, w/v) and heating for 2 min. at 100°. DD and *meso* Isomers were identified as described in the text; LL isomer was identified by the position on the chromatogram. + + +, DAP spot strong, isomer easily identified in hydrolysate (10 μl.); + +, DAP spot visible in hydrolysate (20 μl.); +, isomer identifiable only in electrodialysate (20 μl.); tr., isomer visible only in larger amounts of electrodialysate (30 μl. or more) or in column eluate; + −, isomer stable to action of DAP decarboxylase and L-amino acid oxidase; —, isomer not detected.

Organism	Strain and source*	Grown by†	Diaminopimelic acid isomer		
			meso	LL	DD
Actinomycetales					
Mycobacterium tuberculosis var. *bovis* B.C.G.	‡	See Work & Dewey (1953*a*)	+ + +	—	—
Myco. tuberculosis var. *bovis*	‡	See Work & Dewey (1953*a*)	+ + +	—	—
Myco. avium	‡	See Work & Dewey (1953*a*)	+ + +	—	—
Myco. phlei	NCTC 8151	(1)	+ + +	—	—
Myco. smegmatis	NCTC 3821	(1)	+ + +	—	—
Myco. stercoris	NCTC 8159	(1)	+ + +	—	—
Nocardia asteroides	W.C. 3308 and UCHMS	(2) and local	+ + +	—	—
N. leishmanii	A. 341 LSTM	Local	+ + +	tr.	—
N. corallina	W.C. 3406	(2)	+ + +	—	—
N. rubra	N.R.L. Ottawa 497	Local	+ + +	tr.	—
N. coeliaca	NCTC 2570	Local	+ + +	tr.	—
N. madurae	NCTC	Local	+ + +	tr.	—
N. lutea	NCTC 576	Local	+ + +	tr.	—
Actinomyces (aerobic species)	‡	Local (5)	—	+ + +	—
Streptomyces griseus	W.C. 3463	2	+	+ +	—
Streptomyces fradiae	W.C. 3556B	2	tr.	+ +	—
Streptomyces	MA Clevedon‡	3	—	+ +	—
Streptomyces sp.	Clevedon§	3	+	+ +	—
Streptomyces	XZ-6 Clevedon‡	3	tr.	+ +	—
Streptomyces	X-15 Clevedon‡	3	—	+ +	—
Streptomyces	'Cutler' Clevedon	3	—	+ +	—
Streptomyces	BCC-75 Clevedon‡	3	—	+ +	—
Micromonospora	W.C. 3450	2	+ +	+ +	+ −
Micromonospora	W.C. 3452	2	+ +	+ +	+ −
Micromonospora	LSTM‡	Local (5)	+ +‖	+ +	+ −
Eubacteriales					
Propionibacterium	¶	Local	—	+ + +	—
Bacillus cereus	NRRL 569	Local (5)	+ +	—	—
*B. subtilis***	Cambridge	Local (5)	+ +	—	—
B. megatherium	Lab. strain, Porton	Porton	+ +	+	—
Clostridium butyricum	NCTC 7423	Local (5)	+ +	—	††
Cl. novyi (syn. *Cl. oedematiens*)	Albiston LIPM	4	+ +	—	—
*Cl. acetobutyricum***	UCL	UCL	+ +	—	—
Cl. sporogenes	NCTC 532	Local (5)	+	—	††
Cl. bifermentans	NCTC 2914	Local (5)	+	—	††
Cl. perfringens (syn. *Cl. welchii*) D**	See Work & Dewey (1953*a*)	—	—	+ +	—
Cl. welchii A	S, 107	4	—	+ +	—
Cl. kluyveri	Sheffield‡	UCL	+ +	—	—
Cl. tetani	S, 279	4	+	—	—

* The following abbreviations are used for sources: UCHMS, Department of Bacteriology, University College Hospital Medical School; LSTM, Department of Medical Mycology, London School of Hygiene and Tropical Medicine; Clevedon, Medical Research Council Antibiotics Research Station; Cambridge, Medical Research Council Unit for Chemical Microbiology; LIPM, Lister Institute of Preventive Medicine, Elstree; UCL, Department of Biochemistry, University College, London; Sheffield, Department of Microbiology, University of Sheffield; Porton, M.R.E., Ministry of Supply; NCTC, National Collection of Type Cultures; NRRL, Northern Region Research Laboratory.

† See list of acknowledgements.

‡ These strains have not been further classified.

‖ Also, unidentified amino acid giving same colour reaction, see text.

¶ See text and Table 5 (Antia *et al.* 1957), 6 types of organisms.

** Reported by Hoare & Work (1955*b*).

†† Decarboxylase resistant spot.

§ Tai & van Heyningen (1951).

so low in concentration that it was observed only when the chromatograms were overloaded with *meso* isomer; overloading was not possible unless the diaminopimelic acid was further purified by ion-exchange chromatography. Spots X 1 and 2 show the type of chromatogram obtained with 5 μg. of LL-diaminopimelic acid and 30 or 50 μg. of *meso* isomer; in the absence of other amino acids it is not difficult to detect such low proportions of the LL isomer. However, the reverse process, detection of low amounts of *meso* isomer in the presence of the LL isomer, is not so easy, owing to a tendency of the LL isomer, when present in amounts higher than 30 μg., to 'tail' and so overlap slightly the *meso* spot (X 4). Careful matching against controls will enable this 'tailing' to be distinguished from the true presence of *meso* isomer, as, in this case, the double spot (X 5) presents a club-shaped appearance.

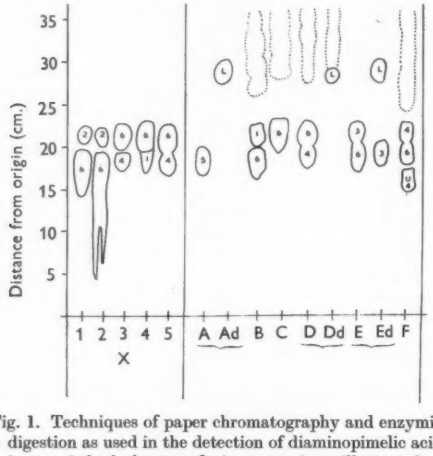

Fig. 1. Techniques of paper chromatography and enzymic digestion as used in the detection of diaminopimelic acid isomers in hydrolysates of micro-organisms, illustrated on members of the Actinomycetales. X 1–5. Chromatograms of LL- and *meso*-diaminopimelic acid, one isomer being present in excess. 1, 5 μg. of LL + 30 μg. of *meso*; 2, 5 μg. of LL + 50 μg. of *meso*; 3, 30 μg. of LL + 5 μg. of *meso*; 4, 50 μg. of LL; 5, 50 μg. of LL + 5 μg. of *meso*. A–F. Chromatograms of either neutral fractions or eluates from Zeo-Karb 215 columns from various Actinomycetales, and of results (denoted by suffix d) of treatment of these fractions with crude diaminopimelic acid decarboxylase as in Methods. A, *Nocardia asteroides* (eluate); B, *Nocardia rubra* (neutral fraction); C, *Streptomyces* MA (neutral fraction); D, *Streptomyces* (Tai & van Heyningen, 1951) (neutral fraction); E, *Micromonospora* W.C. 3452 (eluate); F, *Micromonospora* LSTM (neutral fraction). Chromatography on Whatman no. 1 paper was carried out with overnight irrigation by methanol–water–10 N-HCl–pyridine (80:17·5:2·5:10, by vol.); colours were developed by ninhydrin in acetone (0·1%, w/v) and heating for 2 min. at 100°. Diaminopimelic acid spots (solid outlines containing numbers) and spot U (unknown) gave characteristic olive-green colour fading to permanent yellow; lysine (solid spots designated L) were brownish purple and faded to brown; other neutral amino acids (dotted outlines) were purple, fading rapidly. Numbers on spots indicate colour strength on an arbitrary scale ranging from 1 (just visible) to 6 (maximum intensity).

The only actinomycete examined (an aerobic species) contained a high concentration of LL isomer and no *meso* or DD. This was confirmed with both purified and crude decarboxylase preparations, only the latter producing significant decarboxylation. The streptomyces all contained LL isomer, at lower concentrations than in the actinomycete, and in some strains lesser amounts of *meso* isomer were present; no DD isomer was found (spots C and D).

Two species of *Micromonospora* contained all three isomers; two well-defined diaminopimelic acid spots were observed on chromatograms and, after treatment with crude decarboxylase, a single spot remained in the *meso* position, considerably reduced in intensity from the original spot, while lysine was formed (E and Ed). Control experiments in which either *meso*- or DD-diaminopimelic acid was added to the original solution showed that the added *meso* isomer, but not the added DD compound, was decarboxylated. Similar results were obtained with L-amino acid oxidase, but the residual diaminopimelic acid spot was weaker than after decarboxylase treatment.

Another species of *Micromonospora* (LSTM) contained *meso*-, DD- and LL-diaminopimelic acids, and an unknown amino acid which gave the characteristic diaminopimelic acid colour reaction on methanol–water–HCl–pyridine chromatograms, but which travelled more slowly than diaminopimelic acid (spot F). Added isomers of diaminopimelic acid travelled to their expected positions. This unidentified substance, which is neutral and stable to decarboxylase and peroxide, had previously been observed to travel more slowly than diaminopimelic acid in the phenol direction of two-dimensional chromatograms (Work & Dewey, 1953*b*). No further work has been carried out on this amino acid; it has been observed in hydrolysates made from three different preparations of this organism, which was very slow-growing and required 2–3 months to produce sufficient cells for hydrolysis.

Diaminopimelic acid isomer distribution in certain Eubacteriales

The preliminary results on the LL-diaminopimelic acid content of two species of *Propionibacterium* have been confirmed for other species. The following organisms were examined (the strain numbers being given in Table 5, Antia *et al.* 1957), the number in parentheses indicating the number of strains:

Propionibacterium arabinosus (1), *Pr. jensenii* (3), *Pr. pentosaceum* (2), *Pr. petersonii* (1), *Pr. theonii* (2), *Pr. zeae* (1). All contained similar amounts of LL-diaminopimelic acid, the concentration being sufficiently high for a strong spot to be apparent on chromatography of 10 μl. of hydrolysates. No *meso* isomer could be detected in the neutral fractions; and no diaminopimelic acid remained after treatment with crude decarboxylase. No diaminopimelic acid was found in microbacteria (two strains), *Lactobacillus casei* and *Lb. brevis* (two strains); *Lb. plantarum* (syn. *Lb. arabinosus*) had been previously found to contain *meso*diaminopimelic acid (Hoare & Work, 1955*b*).

Among the Bacillaceae, the finding of LL-diaminopimelic acid in *Clostridium welchii* A (Hoare & Work, 1955*b*, fig. 4) was confirmed with different growth media. All other clostridia examined contained only the *meso* isomer; *Cl. tetani*, previously found to be lacking in diaminopimelic acid (Work & Dewey, 1953*a*), was re-examined in fresh culture and found to contain lower concentrations of diaminopimelic acid than other clostridia. In certain clostridia, indicated in Table 1, the *meso*diaminopimelic acid was found to be partly resistant to decarboxylation; on examination of fresh hydrolysates of the same cultures, this observation could not be repeated, and the amino acid was always completely oxidized by L-amino acid oxidase. The reason for this effect has not been further investigated. *meso*Diaminopimelic acid was previously found in *Bacillus subtilis* and *B. megatherium* and is herewith described in *B. cereus*. However, Mr R. E. Strange, Microbiological Research Department, Porton, Wiltshire, observed small amounts of LL isomer in addition to the *meso* isomer in another culture of *B. megatherium*. At his request we checked this finding, and with his permission are including it among our results.

Diaminopimelic acid content of various intracellular fractions

Fractional centrifuging of the cytoplasmic contents of *Esch. coli* produced three sediments, all free from diaminopimelic acid. Those fractions sedimenting at the lower speeds contained mainly protein with small amounts of DNA; the pellet produced by the highest speed contained RNA, DNA and protein in the proportions 4·3:9·2:59·5. The supernatant liquid remaining after 4 hr. centrifuging at 105 000 *g* contained a low concentration of *meso*diaminopimelic acid. Partially purified diaminopimelic acid decarboxylase from *Aero. aerogenes* did not contain diaminopimelic acid. Only the *meso* isomer of diaminopimelic acid was found in the hexosamine-containing peptide excreted by germinating spores of *B. megatherium* (Strange & Powell, 1954).

The ethanolic extracts of a few species were examined. In *Lb. arabinosus* and *Cl. welchii* (A), no diaminopimelic acid was found in the extracts, either before or after hydrolysis. Approximately equal amounts of *meso*- and LL-diaminopimelic acid were present together in the unhydrolysed ethanolic extracts from *Pr. arabinosus*, *Pr. pentosaceum*, *B. cereus* and *B. subtilis*; the culture of *B. megatherium* in which only the *meso* isomer was found in whole cells had only this isomer free in its ethanolic extract.

In certain organisms, diaminopimelic acid was demonstrable in the ethanolic extracts only after hydrolysis; these include *Pr. jensenii* (*meso* and LL isomers), *Streptomyces* MA (LL isomer), *Cl. cylindrosporum*, *Cl. oedaematiens*, *Myco. smegmatis*, *Myco. tuberculosis* var. *bovis* and *Micromonospora* WC 3452, the last five organisms having only the *meso* isomer. The amounts of diaminopimelic acid in ethanolic extracts varied from about 2 to 10% of that present in whole cells, the highest concentrations being found in bacilli.

DISCUSSION

The original mistaken identification of LL-diaminopimelic acid from streptomyces was due mainly to ignorance of the striking differences between many of the properties of the *meso* and LL isomers. When reference isomers were available, investigation of their physical and chromatographic properties and behaviour towards enzymes revealed the identity of the LL isomer with the unknown amino acid ('A'). Although it is now known that separation of isomers by paper chromatography is more reproducible on Whatman no. 1 paper than on the no. 4 originally used, the difference between the R_F values of 'A' and the *meso*diaminopimelic acid from *C. diphtheriae* was sufficient to differentiate between the two substances. Also the stability of 'A' to partially purified diaminopimelic acid decarboxylase contrasted sharply with the behaviour of diaminopimelic acid from other micro-organisms (Work & Dewey, 1953*a*; Dewey, Hoare & Work, 1954, Table 8). Had the less specific crude preparation of acetone-dried cells been used, this difference in properties would not have been noticed; indeed, when crude cells were later substituted for purified decarboxylase, or when the method of purification of decarboxylase had been altered (Hoare & Work, 1955*b*), the previous findings on the complete stability of 'A' towards decarboxylase could not be repeated. It is now known that the earlier preparations of diaminopimelic acid decarboxylase were the more specific towards the *meso* isomer, whereas crude cells attack both isomers.

The preliminary investigation on the distribution of diaminopimelic acid in the Actinomycetales

(Work, 1953*a*) was left uncompleted pending the identification of amino acid 'A'. The earlier findings have now been mainly confirmed by the improved paper-chromatography method; the only differences are that several isomers were often found together in the same organism. This was not the case among mycobacteria, but in the nocardias and streptomyces minor amounts of a second isomer were sometimes found. The species of *Micromonospora* examined contained roughly similar proportions of LL- and *meso*-diaminopimelic acid. Smaller amounts of DD isomer may also have been present, but there is uncertainty about the identification of this enzyme-resistant form of diaminopimelic acid. It was always found in micromonosporae, but its amount was not consistent in different preparations of the same organism, or when two different enzymes were used to identify it. In one species of this organism, the presence of a third slow spot resembling diaminopimelic acid in its ninhydrin colour reaction is of interest.

In the Actinomycetales, LL-diaminopimelic acid is a common constituent, whereas in the Eubacteriales it is only rarely found. It is possible that phase of growth may influence the overall cellular content of diaminopimelic acid, or even the proportion of isomers. This has not yet been investigated, but it might explain the present finding of *meso*diaminopimelic acid in *Cl. tetani*, whereas previously a culture of unspecified history was found to be free from the amino acid (Work & Dewey, 1953*a*). The Gram-positive cocci provide a group of Eubacteriales from which diaminopimelic acid is nearly always absent, only a few exceptions among the micrococci being known (Cummins & Harris, 1956*a*). The majority of lactobacilli, which are classified in the same family as the streptococci, also appear to be free from diaminopimelic acid. *Lb. arabinosus*, the only member of this genus which we originally examined (Work & Dewey, 1953*a*), is the only species known to contain diaminopimelic acid (Cummins & Harris, 1956*b*); our finding of the *meso* isomer in this organism has been confirmed by isolation (Ikawa & O'Barr, 1955).

One of the major sites for diaminopimelic acid in the bacterial cell is the wall, where it is invariably accompanied by glutamic acid, alanine and glucosamine (Salton, 1953; Cummins & Harris, 1956*a–c*). An insoluble, high-molecular-weight peptide, contained in spore coats of bacilli and liberated in soluble form on standing or on germination, is made up entirely from these same substances (Strange & Powell, 1954; Strange & Dark, 1956; Powell & Strange, 1956). The detailed investigations of Cummins & Harris on the constituents of the cell walls of Gram-positive Eubacteriales and of Actinomycetales have agreed with our results on the distribution of diaminopimelic acid in whole bacterial cells. There appeared to be certain patterns of cell-wall constituents which were often consistent with bacterial taxonomy. This is particularly striking in regard to the distribution of the different isomers of diaminopimelic acid. Thus the cell walls of the mycobacteria, nocardia and corynebacteria were all distinguished by their contents both of large amounts of *meso*diaminopimelic acid and of arabinose and galactose. Cell walls of streptomyces and aerobic actinomyces, on the other hand, contained LL-diaminopimelic acid and glycine, both of which were also constituents of propionibacteria, but which were not usually found in the cell walls of other genera. In organisms lacking in diaminopimelic acid, the cell walls always contained lysine, as in the majority of lactobacilli, where aspartic acid also occurred. The exception, *Lb. arabinosus*, had *meso*diaminopimelic acid, but no lysine or aspartic acid, in its cell walls.

The soluble, non-protein cell contents often, but not invariably, contain diaminopimelic acid, either free (see also Work, 1949; Work & Dewey, 1953*a*; Blass, Lecomte & Macheboeuf, 1951), or bound so as to be apparent only after hydrolysis. The free form may represent the cellular metabolic pool, and its amount and isomer content may depend on the metabolic state of the cells at harvesting. Under certain cultural conditions, the *Esch. coli* mutant 26–26, lacking in diaminopimelic acid decarboxylase, accumulated both LL- and *meso*-diaminopimelic acid in the soluble fraction and excreted them into the culture filtrate (Hoare & Work, 1955*b*). Both isomers were also present in the soluble fractions of some propionibacteria and bacilli, although only one isomer was found in hydrolysates of whole cells of both types of organisms and walls of propionibacteria. We have already suggested that definite cellular fractions may be connected with different functions of diaminopimelic acid (Work & Dewey, 1953*a*); these findings emphasize the wisdom of studying such fractions. The nature of the bound soluble fraction is not known; some of it might be unpolymerized or degraded forms of the peptide units from the cell walls.

No homogeneous bacterial protein free from large amounts of carbohydrate has been found to contain diaminopimelic acid. In all cases where the presence of diaminopimelic acid was reported, either the protein was not shown to be pure, as with shigella endotoxin, where only the most rigorously purified sample was free from diaminopimelic acid (Work & Dewey, 1953*a*), or the 'protein'contained polysaccharide (Seibert, Figueroa & Dufour, 1955) and was probably derived from the cell wall. The total proteins from protoplasts of *B. megatherium* contained no diaminopimelic acid, although the

protoplasts were derived from cells containing it (McQuillen, 1956). The nucleic acid-containing fractions from *Esch. coli* were prepared from lyophilized cytoplasmic contents and cannot therefore be considered as homogeneous. Their freedom from diaminopimelic acid is, however, of interest and indicates absence of contamination from cell-wall fragments.

Diaminopimelic acid is evidently not confined exclusively to bacteria and blue–green algae, as suggested by Work & Dewey (1953*a*). It has been found in *Chlorella ellipsoidia* by Fujiwara & Akabori (1954); we have confirmed this result, but were unable to identify the isomer because of the very low concentration of diaminopimelic acid in the strain examined (Gerneck 211/1*a*, Cambridge Collection of Algae and Protozoa). Systematic examination and fractionation of other micro-organisms unrelated to bacteria may reveal other species containing diaminopimelic acid. The fact that this has not yet occurred suggests that diaminopimelic acid is probably not a common major cellular constituent outside the Schizomycetes.

SUMMARY

1. An unidentified amino acid, previously isolated from streptomyces, was identified as LL-diaminopimelic acid.

2. Among the members of the bacterial order Actinomycetales, the LL isomer was present in hydrolysates of whole cells of streptomyces and an aerobic actinomycete, with small amounts of *meso* isomer in some species. The *meso* isomer occurred in nocardias and mycobacteria, with traces of LL isomer in certain nocardias. In micromonosporae, both isomers were present in similar proportions, with possibly a small amount of DD-diaminopimelic acid.

3. Among selected Gram-positive Eubacteriales, cells of *Clostridium welchii* and propionibacteria contained LL-diaminopimelic acid only, whereas *meso* isomer was present in all other clostridia and bacilli.

4. No diaminopimelic acid was found in nucleic acid-containing fractions which could be sedimented from *Escherichia coli* extracts, but the material remaining in solution after high-speed centrifuging contained *meso* isomer.

5. Ethanolic extracts of cells often, but not invariably, contained diaminopimelic acid, either free or in a bound form liberated by hydrolysis. The isomers present were not always confined to those found in the whole cells from which the extracts were made.

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The Stereoisomers of αε-Diaminopimelic Acid.

3. PROPERTIES AND DISTRIBUTION OF DIAMINOPIMELIC ACID RACEMASE, AN ENZYME CAUSING INTERCONVERSION OF THE LL AND *meso* ISOMERS

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A partially purified preparation of diaminopimelic acid decarboxylase from *Aerobacter aerogenes* was relatively specific for the *meso* isomer of diaminopimelic acid, but the crude enzyme in acetone-dried cells decarboxylated both *meso* and LL isomers, (Hoare & Work, 1955*a*). The initial step in the decarboxylation of LL-diaminopimelic acid was shown, in a preliminary communication, to be conversion into the *meso* isomer (Hoare, 1955). This paper describes experiments leading to recognition of this step and of the enzyme system responsible for it. The enzyme converts either the LL or the *meso* isomer into a mixture of the two forms and is called diaminopimelic acid racemase. Some properties of the enzyme in cell-free systems are described and compared with those of diaminopimelic acid decarboxylase. In order to assess the significance of diaminopimelic acid racemase in bacterial metabolism, its distribution in selected bacteria is examined and compared with that of diaminopimelic acid decarboxylase. An attempt is made to correlate these distributions with the known occurrence of the isomers of diaminopimelic acid in these bacteria (Work & Dewey, 1953; Hoare & Work, 1955*a*, 1957).

Preliminary reports have already presented some of these results (Hoare & Work, 1955*b*, 1956; Work, 1955).

METHODS

The three stereoisomers of diaminopimelic acid were prepared as described (Hoare & Work, 1955*a*, 1957).

Bacteria. Aero. aerogenes was grown as described by Hoare & Work (1955*a*). *Escherichia coli* ACTC 9637 was grown with aeration for 12 hr. at 37° on a similar medium; *Esch. coli* lysine auxotroph 26–26 (Davis, 1952) was grown as described by Work & Denman (1953), except that the L-lysine content of the medium was 30 mg./l. unless otherwise stated. Other bacterial cells were grown on suitable media. All cells were harvested by centrifuging, washed twice with water or saline and immediately dried with acetone at −10°. They were stored at −10° over a desiccant.

Enzyme preparations and tests. All solutions of amino acids and buffers were made in glass-distilled water. Partially purified diaminopimelic acid decarboxylase was prepared from *Aero. aerogenes*, as described by Hoare & Work (1955*a*), and partially purified lysine decarboxylase was obtained from *Esch. coli* B (Hoare, 1956). Decarboxylase assays were carried out at 37° in N_2 in the Warburg apparatus; lysine decarboxylase was estimated at pH 5·5 with L-lysine (13 mM) as substrate; diaminopimelic acid decarboxylase was assayed at pH 6·8 with *meso*diaminopimelic acid (4 mM) as substrate (Dewey, Hoare & Work, 1954). For the estimation of diaminopimelic acid decarboxylase in bacterial cells, acetone-dried cells (40 mg.) suspended in 0·1 M phosphate buffer and pyridoxal phosphate (10^{-5} M) (final vol. 2·5 ml.) were used for each test, and blanks without substrate were included. When the gas output was slight, or the blank value was high, the deproteinized overnight reaction products (0·1–0·3 ml.) were examined for the decarboxylation products, lysine and cadaverine, by paper chromatography (Hoare & Work, 1957) and compared with the blank solutions similarly treated. If no lysine or cadaverine was found in the reaction products, diaminopimelic acid decarboxylase was assumed to be absent. When insufficient cells were available for quantitative tests, the reaction products were examined qualitatively by paper chromatography after incubation of cells with *meso*-diaminopimelic acid at pH 6·8, as described for diaminopimelic acid racemase tests.

Qualitative tests for diaminopimelic acid racemase were carried out on bacteria as follows: acetone-dried cells (3 mg.), suspended in 0·1 M borate buffer, pH 8·5 (0·3 ml.), were incubated with shaking at 37° with either *meso*- or LL-diaminopimelic acid (0·3 mg. in 0·15 ml. of water). Streptomycin was added to a final concentration of 30 μg./ml. to prevent bacterial contamination during overnight incubation. Samples (0·1 ml.) were transferred to ethanol (0·2 ml.) at times 0, 1 and 18 hr. (overnight) and the supernatants examined for diaminopimelic acid isomers by one-dimensional paper chromatography (Hoare & Work, 1957). If, after 1 hr., equal amounts of both LL- and *meso*-diaminopimelic acid were present in the reaction mixture, high racemase activity was indicated. When no isomer interconversion was found after incubation overnight, racemase was considered to be absent. The test was most conveniently carried out with *meso*diaminopimelic acid as substrate, because of the difficulty in detecting slight conversion of the LL into the *meso* isomer (Hoare & Work, 1957). When the cells contained very active diaminopimelic acid decarboxylase, it was necessary to use the LL isomer as substrate, because, in this case, even at pH 8·5, the *meso* isomer was completely decarboxylated to lysine (or cadaverine when lysine decarboxylase was also present) rather than partially racemized to the LL isomer. Under these conditions, racemase activity was indicated by conversion of the LL isomer

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Table 1. *Decarboxylation of* meso- *and* LL-*diaminopimelic acid (DAP) at* pH 6·8 *by acetone-dried bacteria*

Dried cells (40 mg.) were suspended in 2·5 ml. of 0·1 M phosphate buffer containing pyridoxal phosphate (10 μg.) and diaminopimelic acid (2 mg.); atmosphere, N_2: temp., 37°. $Q_{CO_2} = \mu$l. of CO_2/mg./hr. Organisms 1–4 were examined at the beginning of this work, and the others were studied later.

Organism and source*	Q_{CO_2} with *meso*DAP	Q_{CO_2} with LL-DAP	Lag with LL-DAP (min.)	Q_{CO_2} ratio: LL/*meso*
(1) *Aero. aerogenes*, Str. 1; Cambridge	7·8†	2·8	20	0·36
(2) *Esch. coli* B; Carnegie Inst., Washington	12·0†	4·5	10	0·38
(3) *Micrococcus lysodeikticus*; Cambridge	1·2	0·45	40	0·38
(4) *Sarcina lutea*; Cambridge	3·6	2·4	10	0·67
(5) *Staphylococcus citreus*, 36; London	3·3	0·8	0	0·24
(6) *Sporosarcina ureae*; Delft	9·3	0	—	—
(7) *Propionibacterium pentosaceum*; NCTC 8070	0·77	0·51	30	0·66
(8) *Bacillus cereus*; NRRL 569	0	0	—	—

* For definition of contractions see Table 5 and Hoare & Work (1957) Table 1.
† Lysine decarboxylase present.

into lysine or cadaverine; in the absence of racemase no decarboxylation products were detected.

Cell-free extracts containing diaminopimelic acid racemase were prepared at 2° from suspensions of *Esch. coli* 26–26 (1 g.) in 0·01 M phosphate buffer, pH 6·8 (40 ml.). Cells were disintegrated either by hand-grinding with Celite (Dewey *et al.* 1954) or by passage at 12 000 lb./in.^{2} through an apparatus described by Milner, Lawrence & French (1950). Cell residues were removed by centrifuging at 15 000 *g*, and the cell-free extract was either used immediately or stabilized by addition of $HgCl_2$ (0·14 mM) and stored at −10°. Tests of racemase activity were carried out in 0·1 M borate buffer (pH 8·5) with *meso*diaminopimelic acid (0·4 mg./ml.) as substrate. If $HgCl_2$ had previously been added, the enzyme was reactivated by addition of sodium sulphide (final concentration 14 mM) 15 min. before the test was carried out. Solutions were incubated at 37°, and after any three convenient times (e.g. 5, 10 and 20 min.) according to the activity of the enzyme preparation, samples (0·1 ml.) were transferred into ethanol (0·2 ml.) and the supernatants (0·1 ml.) were examined for diaminopimelic acid isomers by paper chromatography.

RESULTS

Recognition of an enzyme system converting LL-*diaminopimelic acid into the* meso *isomer*

Acetone-dried preparations of various bacteria were examined manometrically for decarboxylase activity towards the three stereoisomers of diaminopimelic acid. Organisms known to contain an active decarboxylase attacked both the LL and *meso* isomers (Table 1, nos. 1–4). The DD isomer was not attacked. The LL isomer was always decarboxylated at a lower rate, and there was often a preliminary lag period. The same decarboxylation products, lysine and cadaverine, were formed from both isomers, cadaverine being formed only when lysine decarboxylase was also present (organisms 1 and 2). The results with *Aero. aerogenes* have already been presented in detail in a previous paper, where the relative specificity for the *meso* isomer of the partially purified diaminopimelic acid decarboxylase was also illustrated (Hoare & Work, 1955*a*, Fig. 3 and Table 2). This difference in specificities of the crude and purified enzymes suggested that acetone-dried cells contained an enzyme system which resulted in the ultimate decarboxylation of the LL isomer, and which was almost absent from the partially purified decarboxylase. The presence of two enzymes acting on either the *meso* or the LL isomer was suggested by the variation in the relative rates of decarboxylation of the *meso* and LL isomers shown by different bacteria.

The possibility that decarboxylation of LL-diaminopimelic acid was brought about by L-lysine decarboxylase was discounted by the following facts. Both *meso*- and LL-diaminopimelic acid were decarboxylated by *Sarcina lutea* and *Micrococcus lysodeikticus*, although no lysine decarboxylase was found in either organism. Also, LL-diaminopimelic acid was not decarboxylated by preparations of lysine decarboxylase free from diaminopimelic acid decarboxylase. Such preparations were acetone-dried *Bacillus cadaveris* (Gale & Epps, 1944) or partially purified lysine decarboxylase from *Esch. coli* B (Table 2).

In an attempt to separate the two enzymes acting on diaminopimelic acid in *Aero. aerogenes*, the cell-free extract was subjected to the purification procedure used for *meso*diaminopimelic acid decarboxylase (Hoare & Work, 1955*a*), all fractions being assayed manometrically for decarboxylation of the LL or *meso* isomer. The rate of decarboxylation of the LL isomer was considerably decreased, and the lag period increased, by prolonged dialysis against 10^{-4} M dimercaptopropanol (BAL). Protamine treatment resulted in loss of activity towards the LL isomer, both from the precipitate and the supernatant. This attempt at separation was abandoned and an alternative source of the enzyme attacking only the LL isomer was sought.

Table 2. *Specificity of lysine decarboxylase*

A standard reaction was carried out in Warburg apparatus at pH 5·5, with 6 mg. of lysine hydrochloride or 2 mg. of diaminopimelic acid (DAP). Atmosphere, N_2; temp., 37°.

Enzyme				
Source	Amount (mg.)	Substrate	Time (min.)	Gas output (μl.)
*B. cadaveris** (cells)	20	L-Lysine	5	135
	40	LL-DAP	40	0
	40	*meso*DAP	40	0
Esch. coli B (purified)	1	L-Lysine	10	85
	1	L-Lysine + LL-DAP	10	92
	1	LL-DAP	30	2
	1	*meso*DAP	30	0

* Provided by Dr A. Meister.

For this purpose an examination was made of the lysine auxotroph of *Esch. coli* 26–26 (grown on medium containing 10 mg. of lysine/l.), which lacks diaminopimelic acid decarboxylase (Dewey & Work, 1952). In experiment 1 (Fig. 1), LL-diaminopimelic acid (2 mg.) was incubated for 5·5 hr. at 37° in a Warburg flask containing acetone-dried *Esch. coli* 26–26 (40 mg.) suspended in buffer at pH 6·8 (2·5 ml.) as in a standard decarboxylase assay. A control flask contained boiled cell suspension. No gas evolution occurred in either flask. The reaction mixtures were deproteinized by heating at 100° for 5 min. and centrifuging; similar concentrations of diaminopimelic acid were found in each supernatant solution on examination of portions (0·1 ml.) by paper chromatography in phenol–ammonia. (The system for separation of isomers was not yet developed.) The remainder of each solution was evaporated to dryness at 50°, dissolved in 0·05M phosphate buffer, pH 6·8 (0·5 ml.), and placed in the side bulbs of Warburg flasks, the main compartments of which contained buffered solutions of partially purified diaminopimelic acid decarboxylase. A control flask contained untreated LL-diaminopimelic acid. The LL-diaminopimelic acid which had been previously incubated with unboiled mutant cells was decarboxylated to lysine, while the untreated sample, or that incubated with boiled cells, was not significantly decarboxylated. In Expt. 2, LL-diaminopimelic acid was added to flasks containing *Esch. coli* 26–26, or purified diaminopimelic acid decarboxylase, or a mixture of the two preparations. Significant decarboxylation occurred only when both preparations were present, but in this case there was a preliminary lag compared with the control flask where *meso* isomer was decarboxylated. Previous experiments had demonstrated the complete absence of any decarboxylase activity against *meso* isomer in *Esch. coli* 26–26 when it was

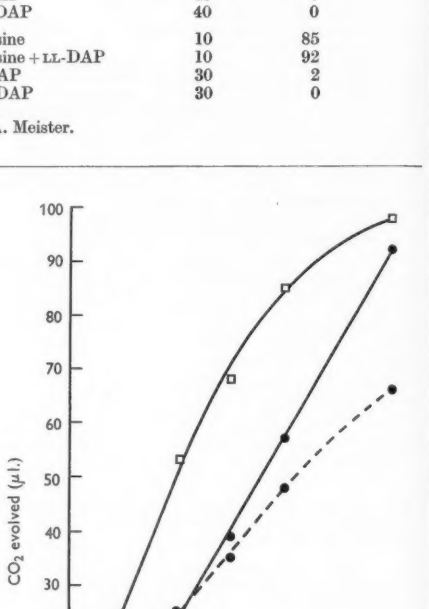

Fig. 1. Manometric demonstration of conversion of LL-diaminopimelic acid into the *meso* isomer by *Esch. coli* 26–26. Incubations were carried out in Warburg flasks at 37°, with enzyme in 0·1M phosphate buffer, pH 6·8 (2 ml.), and diaminopimelic acid (2 mg. in 0·4 ml.) was tipped in from the side arm. Expt. 1 (interrupted lines). ○, LL Isomer tested with partially purified diaminopimelic acid decarboxylase (5 mg.); ●, LL isomer, preincubated with *Esch. coli* 26–26 (40 mg.) for 5·5 hr., boiled, concentrated and tested with decarboxylase. Expt. 2 (solid lines). ○, LL Isomer + decarboxylase (5 mg.); △, LL isomer + *Esch. coli* 26–26 (40 mg.); ●, LL isomer + decarboxylase + *Esch. coli*; □, *meso* isomer + decarboxylase.

examined, either as a suspension of acetone-dried cells or as a cell-free extract; neither did these cell preparations inhibit or enhance activity of crude or purified diaminopimelic acid decarboxylase preparations.

Properties of diaminopimelic acid racemase in Esch. coli 26–26

These experiments suggested that the apparent decarboxylation of LL-diaminopimelic acid was due to two enzymes. One enzyme, referred to below as diaminopimelic acid racemase, converted the LL isomer into the *meso* form; the other enzyme, diaminopimelic acid decarboxylase, acted on the *meso* isomer. It was also shown that *Esch. coli* 26–26 was a source of racemase free from decarboxylase. However, before further investigations on diaminopimelic acid racemase could be made, a method of assay had to be found. Quantitative decarboxylation of the *meso* isomer formed from the LL isomer could not be used, because a preparation of decarboxylase specific to the *meso* isomer could not be conveniently made (Hoare & Work, 1955*a*) and all bacteria examined at that time decarboxylated both isomers or neither one.

Assay. When it was found that the chromatographic solvent methanol–water–pyridine separated LL- and *meso*-diaminopimelic acids (Rhuland, Work, Denman & Hoare, 1955), it was possible that the action of diaminopimelic acid racemase might be demonstrated by paper chromatography. An overlap between lysine and diaminopimelic acid prevented application of the reaction to any systems which contained diaminopimelic acid decarboxylase in addition to the racemase. Addition of hydrochloric acid to the solvent system produced separation of both diaminopimelic acid isomers from lysine and cadaverine, and provided a system for the qualitative assay of racemase, applicable even in the presence of decarboxylase. When an attempt was made to examine, by paper chromatography, the action of the racemase in *Esch. coli* 26–26, it was found that the 'enzyme blank', with no added substrate, gave strong spots of both *meso*- and LL-diaminopimelic acid. This was due to the high intracellular concentration of these diaminopimelic acid isomers which had been built up by *Esch. coli* 26–26 during growth on the medium containing only 10 mg. of lysine/l. (Hoare & Work, 1955*a*). Since no intracellular diaminopimelic acid accumulated when the organism was grown on lysine in concentrations of 30 mg./l. or over, such cells were tested as a source of racemase. They proved satisfactory, being free from decarboxylase and intracellular diaminopimelic acid.

The pH optimum of the racemase in these acetone-dried cells of *Esch. coli* 26–26 was between 8 and 8·5 in both phosphate and borate buffers. The enzyme in acetone-dried cells and cell-free extracts acted on either LL- or *meso*-diaminopimelic acid, producing identical final reaction mixtures containing approximately equal amounts of each isomer, as shown by comparison with known amounts of LL- and *meso*-diaminopimelic acids (Fig. 2). Also, no LL-diaminopimelic acid was formed from the DD isomer, indicating that the DD form was not racemized to the *meso* isomer, since further racemization would have then converted the *meso* into the LL form. No inhibition by the DD isomer was observed, even in concentration five times that of the substrate. When fresh cell-free extracts of these cells were incubated at pH 8·5 with *meso*diaminopimelic acid under the conditions described in methods for the standard racemase test, activity was detectable after 3 min.; after 10 min. the reaction was complete, as judged by the relative intensities of the LL- and *meso*-diaminopimelic acid spots in the reaction mixture.

Activation and inhibition. Freshly prepared extracts were not activated by thiols such as BAL, 2-mercaptoethanol, glutathione (all at 10^{-4} M) or by sodium sulphide (10^{-2} M). Storage at $-10°$ for 24 hr. resulted in a partial loss of activity which was completely reversed by thiols or sodium sulphide;

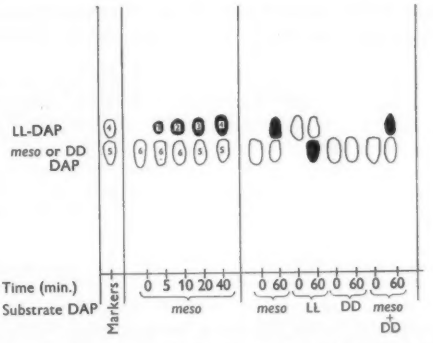

Fig. 2. Racemization of diaminopimelic acid by cell-free extracts of *Esch. coli* 26–26. The extract (0·5 ml.) was prepared as described in Methods; it was incubated in 0·1 M borate buffer, pH 8·5 (0·5 ml.), with diaminopimelic acid isomer (0·5 mg.). Samples (0·1 ml.) were removed at specified times and deproteinized with ethanol (0·2 ml.); the supernatant (0·1 ml.) was examined by chromatography on Whatman no. 1 paper in methanol–water–10 N-HCl–pyridine (80:17·5:2·5:10, by vol.). The spots were developed with ninhydrin in acetone (0·1%, w/v). Black spots indicate reaction products, unshaded spots substrate; numbers on spots indicate colour strength on arbitrary scale varying from 1 (just visible) to 6 (maximum intensity). Markers of *meso*- and LL-diaminopimelic acid (10 μg.).

longer storage resulted in gradually increasing inactivation, which eventually became irreversible after about 4 days. Dialysis produced loss of activity unless carried out against comparatively high concentrations (10^{-3}M) of BAL, when some activity was retained overnight. Additions of pyridoxal phosphate, α-oxoglutarate, boiled extracts of rat liver or *Aero. aerogenes* were all without effect on the activities of fresh, stored or dialysed racemase preparations.

The effects of inhibitors on freshly prepared cell-free extracts of diaminopimelic acid racemase are shown in Table 3. The enzyme is a 'sulphydryl' enzyme, being inhibited by low concentrations of –SH-binding reagents (such as *p*-chloromercuribenzoates or heavy metals) and afterwards reactivated by thiols. This probably accounts for the instability towards storage. Inhibition by carbonyl-binding reagents, such as hydroxylamine, hydrazine and semicarbazide, suggests that a carbonyl group is essential for enzyme activity. The inhibitory action of all these reagents, as well as of *iso*nicotinic acid hydrazide, was prevented or reversed by the presence of thiols (10^{-4}M), but not by pyridoxal phosphate. There was no inhibition by potassium cyanide at concentrations as high as 10^{-2}M, even after pre-incubation of the enzyme with it at pH 6·0, 6·8 or 8·5. 5′-Deoxypyridoxal phosphate was without effect, nor did the chelating agents 8-hydroxyquinoline or ethylenediaminetetraacetic acid inhibit racemization. D- or L-Lysine did not cause inhibition.

Attempts at purification. The instability of diaminopimelic acid racemase in cell-free extracts prevented any fractionation of the free enzyme; for example, even in the presence of 10^{-3}M BAL, activity was lost on attempted fractionation with magnesium or ammonium sulphates or with acetone. At the suggestion of Dr Emil Smith, the enzyme was stabilized as a mercury derivative; the mercury salt (Hg racemase) was easily dissociated by 10^{-2}M sodium sulphide or by 10^{-4}M thiols, and was stable to storage at $-10°$ for 4 months. All fractionation operations were carried out on the Hg racemase, with reactivation by sodium sulphide before testing. The Hg racemase was stable to dialysis against 10^{-2}M phosphate buffer, pH 5·8–6·8 at $+2°$ for 24 hr., but lost considerable activity on 3 days' dialysis and was not then reactivated by pyridoxal phosphate.

Purification by acetone fractionation of protamine-treated extracts, the method used for diaminopimelic acid decarboxylase, could not be used on fresh racemase or Hg racemase. Precipitation of nucleic acid from extracts containing Hg racemase by excess of protamine sulphate (2 mg./ml.) resulted in loss of activity from the solution, and the precipitate could not be redissolved. By halving the amount of protamine used, about half the nucleic acid and protein were precipitated, as indicated by change in optical densities at 280 and 260 mμ, and the solution retained all its activity. Acetone fractionation of this solution did not produce clear-cut separations, probably owing to the presence of nucleic acids. All activity was lost by precipitation with ammonium sulphate. The use of streptomycin (10 mg./ml. of extract) as a nucleic acid precipitant also removed all racemase from solution, but in this case the precipitate could be redissolved in borate buffer (pH 8·5) and all activity recovered. Acidification of Hg racemase solutions to pH 4·8 produced a heavy precipitate, which, after washing with water, could be redissolved by raising the pH to 6·0 or above, and contained full racemase activity. Neither acid nor streptomycin precipitation removed any nucleic acid from the racemase, as shown by the constancy of the ratios of optical densities at 280 and

Table 3. *Effect of inhibitors on diaminopimelic acid racemase in fresh cell-free extract of* Esch. coli 26–26

The test was carried out as described in Methods. In the absence of inhibitor, racemization was complete in 20 min.; complete inhibition was shown by the presence of only one isomer of diaminopimelic acid in the reaction mixture after the time shown.

Inhibitor	Concn. (M)	Reaction time (min.)	Inhibition
Mercuric chloride	10^{-4}	60	Complete
Cupric sulphate	10^{-5}	60	Complete
p-Chloromercuribenzoate	10^{-3}	60	Complete
EDTA	10^{-3}	120	None
Oxine	10^{-4}	40	None
Cyanide	10^{-2}	40	None
Hydroxylamine	10^{-3}	120	Complete
Hydrazine	10^{-2}	120	Partial
Semicarbazide	10^{-2}	120	Partial
*iso*Nicotinic acid hydrazide*	10^{-3}	120	Partial
	10^{-2}	120	Complete
Deoxypyridoxal phosphate	10^{-4}	120	None

* Pre-incubated with enzyme for 30 min.

260 mμ. These facts suggest that in *Esch. coli* 26–26 diaminopimelic acid racemase may exist in close association with nucleic acid.

The pH optimum of the racemase after storage as the Hg salt was very similar to that of the enzyme tested in acetone-dried cell suspensions, being between about 7·7 and 8·3. The alkalinity of sodium sulphide made it unsuitable for reactivation of the Hg racemase for the pH investigations, so BAL (10^{-4} M) was used. This proved to be slightly acid, but the pH of the final reaction mixture was always checked. Slight racemase activity was observed at pH of 9·1 or 6·0, and at pH 6·8 the activity was only slightly lower than at pH 8·3. Unfortunately the method of estimation does not allow a true pH–activity relationship to be established.

Diaminopimelic acid racemase, purified by streptomycin precipitation, was examined for alanine racemase activity as follows. The streptomycin-precipitated Hg racemase (10 mg.) dissolved in 0·1 M aminotrishydroxymethylmethane (tris) buffer, pH 7·3 (1 ml.), was mixed with 0·1 M sodium sulphide (0·7 ml.). A portion (0·4 ml.) was tested over 40 and 100 min. with *meso*diaminopimelic acid (0·2 mg.) for diaminopimelic acid racemase. To the remainder, L-alanine (0·175 mg.) and pyridoxal phosphate (10 μg.) were added; a portion of the reaction mixture was removed immediately as a control and immersed for 5 min. in boiling water. The remaining preparation was incubated at 37° for 40 or 100 min. and then boiled. Protein was removed by centrifuging from the control and incubates, and each supernatant (0·4 ml.) was treated with a dialysed solution (0·15 ml.) of L-amino acid oxidase from *Crotalus adamanteus* (rattlesnake) venom (20 mg. of dried venom per ml. of 0·1 M tris buffer, pH 7·3). Both mixtures were gassed with oxygen, corked and shaken at 37° for 3 hr., after which samples (0·2 ml.) were deproteinized with ethanol and examined by one-dimensional paper chromatography. The results showed that after 40 min. incubation with racemase preparation a small amount of L-alanine had been converted into the D isomer, since there was a faint alanine spot left after treatment with L-amino acid oxidase, whereas in the boiled control sample all the alanine had disappeared after incubation with oxidase. Considerably more D-alanine was apparent when the incubation period was extended to 100 min. but racemization was not yet complete. Diaminopimelic acid was completely racemized in 40 min. by the racemase preparation. This showed that, although alanine racemase was present, it was less active than diaminopimelic acid racemase.

Comparison of inhibition of diaminopimelic acid racemase and decarboxylase in normal bacterial cells

Diaminopimelic acid racemase resembled diaminopimelic decarboxylase in being inhibited by most reagents which bind either thiol or carbonyl groups; the racemase differed from decarboxylase in its stability to potassium cyanide and in its

Table 4. *Comparative effects of inhibitors on diaminopimelic acid decarboxylase and racemase acting simultaneously*

Expt. *A*, *Esch. coli* ACTC 9637, 10 mg. as cell suspension. Expt. *B*, *Aero. aerogenes*, 5 mg. as cell-free extract. Incubations were carried out at 37° in 0·1 M buffer (0·6 ml.) at the pH shown, with 0·2 mg. of either *meso*- or LL-diaminopimelic acid (DAP). Samples (0·2 ml.) were transferred at times shown to 0·4 ml. of ethanol. A portion (0·1 ml.) of supernatant liquid was examined by paper chromatography with methanol–water–pyridine–HCl solvent. Reaction products are indicated by strength, judged by eye, of ninhydrin spots, varying from + + + (maximum strength) to + – (just visible); – indicates not visible.

Expt.	Inhibitor (M)	pH	Substrate DAP	Time (min.)	Reaction products: *meso*DAP	LL-DAP	Lysine
A	.	8·5	LL	15	+	+	+ +
	.	8·5	LL	60	–	–	+ + +
	.	6·8	*meso*	15	+ –	+ –	+ +
	.	6·8	*meso*	60	–	–	+ + +
	KCN, 10^{-2}	8·5	LL	15	+	+	+ –
	KCN, 10^{-2}	8·5	LL	60	+	+	+
	KCN, 10^{-2}	6·8	*meso*	15	+	+	+ –
	KCN, 10^{-2}	6·8	*meso*	60	+ –	+ –	+ +
	$CuSO_4$, 10^{-5}	8·5	LL	15	–	+ +	–
	$CuSO_4$, 10^{-5}	8·5	LL	60	+ –	+ +	–
	$CuSO_4$, 10^{-5}	6·8	*meso*	15	+ +	–	+ –
	$CuSO_4$, 10^{-5}	6·8	*meso*	60	+ +	–	+ –
B	.	8·5	LL	120	+	+	+ –
	.	6·8	*meso*	120	+ –	+ –	+ +
	NH_2OH, 10^{-3}	8·5	LL	120	–	+ + +	–
	NH_2OH, 10^{-3}	6·8	*meso*	120	+ + +	–	–
	NH_2OH + BAL, 10^{-3}	8·5	LL	120	+	+	–
	NH_2OH + BAL, 10^{-3}	6·8	*meso*	120	+	+	–

insensitivity to carbonyl-binding reagents acting in the presence of added thiols. It was possible that these differences might have been due to the different sources of the two enzymes, since the racemase was obtained from cells of a mutant *Esch. coli*, the abnormal constituents of which might have interfered with added inhibitors. Accordingly, the two enzymes were investigated simultaneously in preparations of normal bacterial cells, either *meso*-diaminopimelic acid at pH 6·8 or LL-diaminopimelic acid at pH 8·5 being used as substrate (Table 4). In the absence of inhibitors, the net result was conversion of either isomer of diaminopimelic acid into lysine; this was due to the overlapping pH ranges of the two enzymes and to the fact that the equilibrium mixture produced by the racemase was continually changed by decarboxylation of the *meso* isomer. However, by examination of reaction products at specified times, it was possible to investigate inhibition of either enzyme. *Esch. coli* 9637, the parent strain of mutant *Esch. coli* 26–26, at pH 8·5 completely converted LL-diaminopimelic acid into lysine in 1 hr., whereas after 15 min. the reaction products included both *meso*diaminopimelic acid and some unchanged LL isomer. At pH 6·8, *meso*-diaminopimelic acid was mostly decarboxylated to lysine, but a small amount had been racemized to the LL isomer after 15 min. In the presence of 10^{-2}M potassium cyanide racemase was not inhibited, as shown by the interconversion of the diaminopimelic acid isomers at either pH, but the decarboxylase was partially inhibited, since lysine formation was reduced, especially after 15 min. In contrast, cupric sulphate (10^{-5}M) caused almost complete inhibition of racemase and a considerable inhibition of decarboxylase. In cell-free extracts of *Aero. aerogenes*, both racemase and decarboxylase were completely inhibited by 10^{-3}M hydroxylamine; with BAL (10^{-3}M) also present, racemization, but not decarboxylation, occurred.

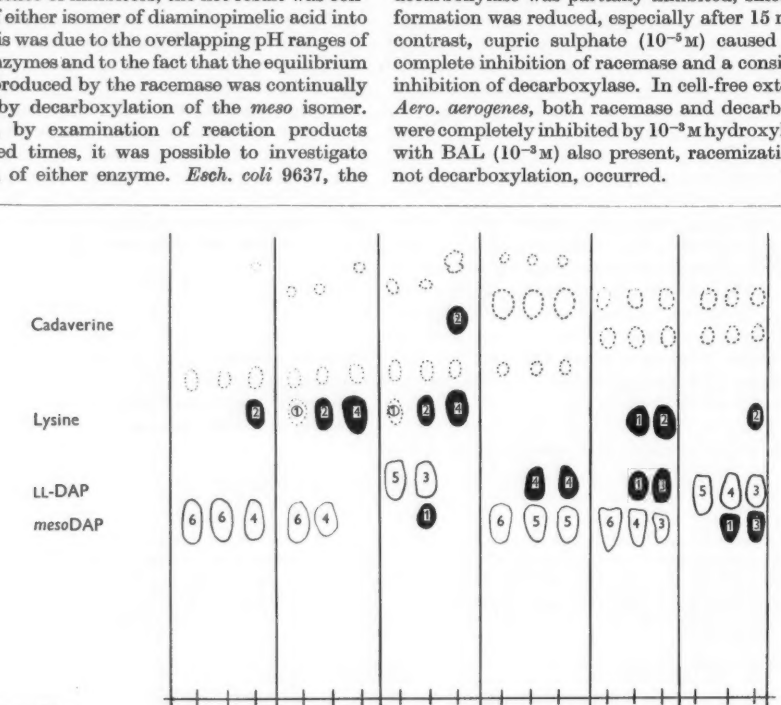

Fig. 3. Some typical tests for diaminopimelic acid racemase on various species of bacteria. Cells (3 mg.) were incubated at 37° with one isomer (LL or *meso*) of diaminopimelic acid (DAP) as substrate (3 mg. in 0·15 ml. of water) in 0·1M borate buffer, pH 8·5 (0·3 ml., containing 30 μg. of streptomycin). Samples (0·1 ml.) were taken at the times specified and deproteinized with ethanol (0·2 ml.); the supernatant (0·1 ml.) was examined by chromatography in methanol–water–10N-HCl–pyridine (80:17·5:2·5:10, by vol.) on Whatman no. 1 paper. The spots were developed with ninhydrin in acetone (0·1%, w/v). Dotted spots indicate intracellular amino acids (unrelated to test). Unshaded spots indicate substrate, and black spots indicate reaction products; numbers on spots indicate colour strength of spot on an arbitrary scale, varying from 1 (just visible) to 6 (maximum intensity). The units of racemase activity, as in Table 5, are: + +, complete racemization in 1 hr.; +, partial racemization in 1 hr.

* With LL-DAP reaction products also identical at 1 and 18 hr.

Table 5. *Diaminopimelic acid decarboxylase and racemase contents of various bacteria in relation to diaminopimelic acid (DAP) isomer distributions*

Enzyme tests were as described in Methods. The contents of decarboxylase are indicated as Q_{CO_2} (= μl./mg./hr.) or, when detected by chromatography, as + or sl. Racemase contents, indicated as degree of racemization: + +, complete in 1 hr.; +, partial in 1 hr.; sl., only detectable after 18 hr.

Organism	Strain and source*	Growth conditions: Medium†	Temp.	Time (days)	Decarboxylase (Q_{CO_2})	Racemase	DAP
Micrococcaceae							
Staphylococcus aureus	Oxford	A	37°	3	0·8	.	0
	Oxford	D	37	2	2·9	+ +	0
Staph. citreus	36 (London)	C	37	0·75	3·2	+	0
Micrococcus citreus	NCTC 7731	C	37	0·75	sl.	sl.	*meso*
	NCTC 7731	C	37	1	0	0	.
M. varians	NCTC 7281	C	37	1·3	0·8	+ +	*meso*
M. lysodeikticus	Cambridge	A	25	3	3·0	+ +	0
	Cambridge	B	25	3	3·8	.	0
Sarcina lutea	Cambridge	A	25	3	2·7	+ +	0
	Cambridge	B	25	3	5·8	+ +	0
Sporosarcina urea	Delft	E	Room	2	6·9‡	0	0
	Edinburgh	E	Room	2	3·1	0	0
Lactobacillaceae							
Strep. faecalis	R Cambridge	G2	37	4	0	0	0
	ATCC 9790	G1	37	3	0	0	0
	ATCC 9790	H	37	3	0	0	0
Strep. lactis	NCIB 8586	J	37	1	0	0	0
Leuconostoc mesenteroides	P 60 Cambridge	G2	37	1–5	0	0	0
	NCIB 6990	K	25	1	0	0	0
Ln. citrovorum	NCIB 7837	K	25	1	0	0	0
Microbacterium lacticum	NCTC 8541	G1	30	4	1·1	+	0
Lactobacillus plantarum (syn. *Lb. arabinosus*)	ATCC 8014	G2	37	1–5	0	+	*meso*
Lb. casei	ATCC 7469	G2	37	2	0·5	sl.	0
Lb. brevis	L3 Birmingham	L	30	2	0	sl.	0
	L4 Birmingham	L	30	1	0	0	0
Propionibacterium arabinosum	NCTC 5958	M	30	3	1·4	+	LL
Pr. jensenii	NCTC 8069	N	30	3	+	+ +	LL
	NCTC 8071	M	30	3	sl.	sl.	LL
Pr. pentosaceum	NCTC 8070	N	30	3	1·5	+ +	LL
	van Niel	M	30	3	2·7	+ +	LL
Pr. petersonii	NCTC 5962	M	30	4	+	+	LL
Pr. thoenii	NCTC 5966	M	30	4	sl.	0	LL
	NCTC 8072	M	30	3	sl.	0	LL
Pr. zeae	NCTC 5967	N	30	3	3·3	+ +	LL
Bacillaceae							
Bacillus subtilis	Cambridge	F	37	.	0	+	*meso*
B. cereus	NRRL 569	F	37	1	0	+ +	*meso*
Clostridium butyricum	NCTC 7423	P	37	2	0	0	*meso*
Cl. novyi (syn. *Cl. oedematiens*)	Albiston LIPM	Q	35	1	0	0	*meso*
	Albiston LIPM	Q	35	3	0	0	*meso*
Cl. acetobutyricum	UCL	P	.	.	0	.	*meso*
Cl. sporogenes	NCTC 532	P	37	3	0	0	*meso*
Cl. bifermentans	NCTC 2914	P	37	1	0	+ +	*meso*
Cl. perfringens (syn. *Cl. welchii*) A	S107 LIPM	R	35	0·35	0	+	LL
	S107 LIPM	P	35	0·7	0	+	LL
Cl. tetani	S279 LIPM	P	35	3	2·6	sl.	*meso*
Actinomycetales							
Mycobacterium phlei	NCTC 8151	S	37	7	0·2	+	*meso*
Myco. stercoris	NCTC 8159	S	37	7	0·5	+	*meso*
Myco. smegmatis	NCTC 3821	S	37	7	0·2	+	*meso*
Actinomyces	Aerobic sp.	F	30	5	0	0	LL
Nocardia asteroides	UCHMS	G2	37	2	0·7	+	*meso*

* For most abbreviations see Hoare & Work, 1957, table 1. Others are: London, Dept. of Bacteriology, London Hospital. Edinburgh, Edinburgh and East of Scotland College of Agriculture. Birmingham, Dept. of Applied Biochemistry, The University. Delft, Microbiological Laboratory, Technical High School. ATCC, American Type Culture Collection.

† Growth media were as follows: (Figures in parentheses indicate %, w/v, except for R and S). A, Nutrient agar; B, glucose (1) nutrient agar; C, serum (5), glucose (1), nutrient agar; D, CCY agar (Gladstone & Fildes, 1940); E, urea (0·5), peptone (1), Lab Lemco (1); F, nutrient broth; G1, glucose (1), nutrient broth; G2, glucose (2), nutrient broth; H, blood (1), glucose (1), nutrient broth; J, glucose (0·5), tryptone (1), yeast extract (1), K_2HPO_4 (0·5); K, glucose (0·5), tryptone (1), yeast extract (1), K_2HPO_4 (0·5), tomato juice (20); L, wort broth; M, yeast extract (0·2), sodium lactate (2); N, yeast extract (0·2), sodium lactate (2), salts; P, cooked meat broth; Q, sodium sulphate meat broth; R, van Heyningen (1948); S, Dubos & Davis (1946).

‡ Increased by storage.

Distribution of diaminopimelic acid racemase and decarboxylase in certain bacteria

The activities of diaminopimelic acid racemase and decarboxylase were determined in various Micrococcaceae, Lactobacteriaceae, Bacillaceae and a few Actinomycetales. Some examples of typical racemase tests on acetone-dried organisms are shown in Fig. 3. The results are summarized in Table 5, and presented with the diaminopimelic acid isomer content of the organisms, reported in the preceding paper (Hoare & Work, 1957). Since no systematic investigation of the effect of growth conditions on enzyme activity was made, the conditions of growth have been included in the table. Both enzymes were present in many, but not all, organisms examined. There was no relation between racemase content and isomer distribution; thus *Clostridium welchii* had a racemase activity similar to that of other clostridia, although it differed in containing only LL-diaminopimelic acid. Various degrees of racemase activities were found in other clostridia, all of which contained *meso*diaminopimelic acid. With the exception of *Cl. tetani*, no clostridia or bacilli examined contained measurable amounts of diaminopimelic acid decarboxylase, there being no detectable decarboxylation of either the LL or *meso* isomer (Table 1, no. 8).

Many of the Micrococcaceae, which contained no diaminopimelic acid, had very active diaminopimelic acid racemase and decarboxylase. Two strains of *Micrococcus*, examined because they were found by Cummins & Harris (1956) to have diaminopimelic acid in their cell walls, contained the *meso* isomer, but had less racemase and decarboxylase than the staphylococci. Considerable variations in the decarboxylase contents of Micrococcaceae resulted from changes of culture medium, the richer the medium in nutrients and the higher the cell yield, the higher were the resultant decarboxylase levels. Since low yields were obtained of the two micrococci which contained diaminopimelic acid, it is possible that the low enzyme activities were a consequence of poor growth. *Sporosarcina ureae* was without racemase activity, although high decarboxylase levels were found in both strains; the activity rose on storage of cells at $-10°$, probably owing to loss of an enzyme competing for the substrate. No detectable decarboxylation of the LL isomer occurred (Table 1, no. 6).

The Lactobacteriaceae fell into two main groups, the coccal organisms—streptococci and leuconostocs—which contained no detectable racemase, decarboxylase or diaminopimelic acid; and the rod-shaped bacteria, nearly all of which contained one or both of these enzymes and, in certain cases, also diaminopimelic acid. Thus the propionibacteria, all having high levels of LL-diaminopimelic acid, contained levels of racemase varying from very high to undetectable. All propionibacteria showed decarboxylase activities when tested chromatographically; quantitative estimation of activity was often difficult to carry out, because of very high endogenous gas production; this did not occur with other organisms. Among the closely related lactobacilli and microbacteria, only *Lb. arabinosus* contained diaminopimelic acid (the *meso* isomer), yet most of them contained racemase or decarboxylase. Among the Actinomycetales, the three mycobacteria examined contained low racemase and decarboxylase activities, as did *Nocardia asteroides*. An aerobic actinomycete, containing LL-diaminopimelic acid, had no detectable racemase or decarboxylase.

DISCUSSION

Several bacterial amino acid racemases are known; they all act on both optical isomers and produce racemic mixtures. Alanine racemase is widely distributed among bacteria, but has not been found in other micro-organisms or in animal tissues; its pH optimum is about 8·5; it requires pyridoxal phosphate for activation, even in acetone-dried cell preparations (Wood & Gunsalus, 1951; Marr & Wilson, 1954). Glutamic acid racemase in *Lb. arabinosus* also has pH optimum of 8, but there are conflicting reports as to its requirement for pyridoxal phosphate (Ayengar & Roberts, 1952; Narrod & Wood, 1952). Threonine racemase in *Esch. coli* requires ATP or AMP as a cofactor, and there is no mention of a pyridoxal phosphate requirement (Amos, 1954). Racemization *in vitro* of amino acids was produced by heating at pH 10 with pyridoxal and metal salts; at pH 5, transamination, but no direct racemization, occurred (Olivard, Metzler & Snell, 1952).

Any consideration of racemization of diaminopimelic acid is complicated by the fact that the amino acid has two asymmetric carbon atoms; it has three isomeric forms, since the *meso* form is symmetrical about the γ-carbon atom. The enzyme which catalyses the interconversion of *meso* and LL-diaminopimelic acids has been called diaminopimelic acid racemase. The reaction catalysed may be written diagrammatically as

$$\begin{array}{ccc} \text{D} & \rightleftharpoons & \text{L} \\ | & & | \\ \text{L} & & \text{L} \end{array}$$

Thus the intramolecular rearrangement produced by the enzyme involves the groups attached to only one of the asymmetric carbon atoms; this carbon may be in the D or the L configuration. The other asymmetric carbon atom must be in the L configuration, since the DD isomer is not attacked. Although

no accurate measurements were made on the composition of the equilibrium mixture, paper chromatography showed that it consists of an approximately equal mixture of the LL and *meso* isomers. Since the reaction product is not a racemic mixture of optical antipodes, it might be argued that the enzyme is not a racemase in the usual sense of the word; true racemization does, apparently, occur at one of the two asymmetric centres. The properties of diaminopimelic acid racemase, particularly the alkaline pH optimum, are similar to those of the other bacterial amino acid racemases. The possibility that it might be the same enzyme as one of the other racemases with a broad specificity has not been entirely eliminated, even for alanine racemase; however, alanine racemase occurs in streptococci (Wood & Gunsalus, 1951) from which diaminopimelic acid racemase is missing, so it is probable that the two enzymes are different.

*meso*Diaminopimelic acid is a substrate for the two enzymes, diaminopimelic acid racemase and decarboxylase. Until this wide survey of distribution of these two enzymes was undertaken, we were unable to determine whether diaminopimelic acid decarboxylase had an absolute specificity for the *meso* isomer or whether it also acted very slowly on the LL isomer. Fractionated decarboxylase preparations had slight activity towards the LL isomer (Hoare & Work, 1955*a*), and no bacteria had been then found which possessed an active decarboxylase but no racemase. However, the *Sporosarcina* species, on which we report in this paper, contain no diaminopimelic acid racemase and have strong decarboxylase activity towards only *meso*diaminopimelic acid (Table 1). The absolute specificity of the decarboxylase is thus proved in this organism. It is interesting to note that the point of attack of both racemase and decarboxylase on *meso*diaminopimelic acid is at the asymmetric centre in the D configuration, the products being LL-diaminopimelic acid and L-lysine respectively. The L configuration for the other asymmetric centre appears to be necessary for enzyme activity. It is therefore not surprising that D-lysine and DD-diaminopimelic acid do not inhibit either enzyme. As far as specificity to homologues is concerned, diaminopimelic acid decarboxylase has no activity against other diaminodicarboxylic acids (Dewey *et al.* 1954); the specificity of the racemase is not known, because the method of testing activity cannot be applied to other amino acids as their isomers cannot be resolved by paper chromatography. Both diaminopimelic acid racemase and decarboxylase are sulphydryl enzymes; the racemase is the more sensitive to oxidation but can be stabilized as its mercury salt. Direct proof is not available as to whether pyridoxal phosphate is involved in racemase function. The inhibition of racemase by aldehyde-binding reagents resembles that of diaminopimelic acid decarboxylase, which is activated by pyridoxal phosphate (Hoare, 1956). The stability of racemase to potassium cyanide cannot be explained, since this reagent is generally an efficient inhibitor of enzymes activated by pyridoxal phosphate. The reversal by thiols of the inhibition of racemase by *iso*nicotinic acid hydrazide and carbonyl-binding reagents is also unexplained; it does not occur with diaminopimelic acid decarboxylase. A similar reversal of *iso*nicotinic acid hydrazide inhibition of cysteine sulphinic acid decarboxylase was noted by Davison (1956); this enzyme is a 'sulphydryl' enzyme requiring pyridoxal phosphate.

In the study of the distribution of these two enzymes known to metabolize diaminopimelic acid, only a few families of Gram-positive Eubacteriales were examined in detail. These families were selected because the commonly occurring *meso*-diaminopimelic acid was not invariably present; thus in the Micrococceae and the majority of Lactobacteriaceae (propionibacteria excepted) no diaminopimelic acid occurs, while among the Bacillaceae an exception was *Cl. welchii*, with its content of LL-diaminopimelic acid. It was hoped that some clue might be obtained as to the reason for this distribution, but this was not found, except in the Streptococcaceae. The enzyme contents of organisms containing *meso*diaminopimelic acid did not differ in any one respect from those containing the LL isomer.

No full investigation of the effect of phase of growth on enzyme activities has been made; it is known that different species differ markedly in this respect, as with diaminopimelic acid decarboxylase contents of *Esch. coli* and *Aero. aerogenes* (Dewey *et al.* 1954). In the staphylococci, improvement in growth medium raised the activity of diaminopimelic acid decarboxylase. Since it is possible that some losses of enzyme activities might have occurred through the use of unsuitable growth conditions, it might be wiser to place more reliance on the positive results of enzyme activity rather than on the odd negative anomaly. However, when nearly all members of a family or genus lack a given enzyme, it is felt that more importance can be attributed to the results. Thus, among the Bacillaceae, diaminopimelic acid decarboxylase is absent from all except *Cl. tetani* (*B. cadaveris* was not included in Table 5; it had a very active lysine decarboxylase but no diaminopimelic acid decarboxylase, see Table 2). The Streptococcaceae also provide a consistent group, containing neither diaminopimelic acid nor its racemase or decarboxylase; possibly in these nutritionally exacting cocci, diaminopimelic acid plays no part in cellular metabolism. These Streptococcaceae are sharply differentiated from the other Eubacteriales, even

from the related lactobacilli and propionibacteria, in all of which diaminopimelic acid appears to be a metabolite, judged by its presence in the cell or by the activity of enzymes attacking it. The majority of the other Gram-positive cocci in the family Micrococcaceae also lack diaminopimelic acid; the presence of active racemase and decarboxylase suggests that these organisms may utilize diaminopimelic acid as a transitory metabolite but do not usually incorporate it into their cell walls. The fact that two exceptional micrococci contain diaminopimelic acid (Cummins & Harris, 1956) supports the hypothesis that diaminopimelic acid is a metabolite in Micrococcaceae.

One known metabolic function of diaminopimelic acid in *Esch. coli* is to act as a precursor of lysine through the action of diaminopimelic acid decarboxylase (Work, 1955). The racemase is known also to be functional in the metabolism of this organism, since the lysine auxotroph, *Esch. coli* 26–26, blocked at the decarboxylase step, accumulates a mixture of equal amounts of LL- and *meso*-diaminopimelic acids. Also, the diaminopimelic acid auxotroph, *Esch. coli* 173–25, responds equally well to LL- or *meso*-diaminopimelic acids (Hoare & Work, 1955*a*).

This study of the distribution of diaminopimelic acid racemase has not answered the questions of the function of the racemase in metabolism, or as to which isomer of diaminopimelic acid is synthesized first. The racemase is even more widely distributed among bacteria than is the decarboxylase. It is possible that LL-diaminopimelic acid might be synthesized first from precursors in the LL configuration; the *meso* isomer, found in most bacterial cells, may be subsequently formed by racemization. The finding of only the LL isomer of diaminopimelic acid in whole cells of *Cl. welchii* and propionibacteria is not due to lack of racemase. Since most propionibacteria also contain decarboxylase, it is possible that the racemase in these organisms is acting simply in the enzyme sequence necessary for lysine formation. The intracellular soluble amino acids of propionibacteria resemble a racemase equilibrium mixture in their content of both *meso*- and LL-diaminopimelic acid (Hoare & Work, 1957). In the clostridia, with no diaminopimelic acid decarboxylase, lysine is probably not normally formed endogenously; if it is, it may be derived from some source other than diaminopimelic acid.

SUMMARY

1. The enzyme diaminopimelic acid racemase converts either LL- or *meso*-diaminopimelic acid into a mixture of the two isomers. It has no action on DD-diaminopimelic acid.

2. This enzyme, acting in sequence with diaminopimelic acid decarboxylase (which is specific for *meso*diaminopimelic acid), is responsible for the apparent decarboxylation of LL-diaminopimelic acid by many acetone-dried bacteria.

3. The properties of diaminopimelic acid racemase have been examined in cell-free extracts of *Esch. coli* 26–26, which are free from decarboxylase. The pH optimum is about 7·7–8·3. The enzyme has a sensitive sulphydryl group, but can be stabilized as an inactive mercury complex and subsequently reactivated. It is not activated by pyridoxal phosphate, but is inhibited by carbonyl-binding reagents; this inhibition is reversed by thiols.

4. Diaminopimelic acid racemase is even more widely distributed among bacteria than is the decarboxylase. The distribution of these enzymes bore no relation to the occurrence of the isomers of diaminopimelic acid. Only among the Streptococcaceae were both these enzymes and diaminopimelic acid absent. Decarboxylase was absent from most Bacillaceae, but racemase was usually present.

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Note added in proof. Since this paper went to press diaminopimelic acid decarboxylase has been found in *B. sphaericus* (cf. p. 457).

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Connective Tissue Growth Stimulated by Carrageenin

2. THE METABOLISM OF SULPHATED POLYSACCHARIDES*

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Robertson & Schwartz (1953) have shown that the subcutaneous injection of carrageenin in guinea pigs causes the formation of large amounts of easily separated connective tissue. In the course of preliminary experiments, which have been briefly reported by Jackson (1956), this method has been found to provide a very convenient experimental model for biochemical studies on connective-tissue formation and removal. The new tissue so formed is readily separated from surrounding skin, fascia and muscle and amounts of the order of 10–20 g. of granuloma tissue/guinea pig are regularly obtained in the early phase of the response to a single injection of 50 mg. of carrageenin. In the earlier paper of this series some observations on the formation and removal of collagen in the carrageenin-induced granuloma are reported (Jackson, 1957). The present paper is concerned with the metabolic behaviour of sulphated polysaccharides from the same granulation tissue, and from skin and rib cartilage of normal animals.

It has been shown by Layton (1950) that there is an increased uptake of sulphate *in vitro* by granulation tissue formed after muscle injury. Kodicek & Loewi (1955) studied the uptake of [^{35}S]sulphate by mucopolysaccharides of forming connective tissue after tendotomy in guinea pigs. They found that the uptake of ^{35}S reached a maximum from the 7th to 15th days and returned to low values on the 23rd day after operation. The radioactivities measured by them relate to total organic [^{35}S]sulphate in the granulation tissue after preliminary extraction with Ringer solution, and therefore loss of unknown amounts of soluble organic [^{35}S]sulphate in this fraction. Some 67% of the [^{35}S]sulphate in the Ringer-washed granulation tissue was shown to be present in an alkali-extractable polysaccharide having a mobility on paper ionophoresis similar to chondroitin sulphuric acid and which was metachromatic to toluidine blue. There remained some 25% of [^{35}S]sulphate activity unaccounted for, of which apparently only a small part is contained in residue material. But paucity of extracted material made further characterization impossible. In the present experiments the uptake of [^{35}S]sulphate into three fractions of the granuloma and other tissues of guinea pigs has been studied. The first fraction is that extracted along with neutral salt-soluble collagen, i.e. by neutral 0·2 M sodium chloride. The second fraction is brought into water or dilute salt solution by digestion with activated papain, which destroys the protein linkages. Finally, there is an organic sulphate fraction in residual material which resists dissolution even by papain digestion and which will be referred to as residue sulphate.

* Part 1: Jackson (1957).

METHODS

Treatment of animals

Guinea pigs were treated as described in the preceding paper, Jackson (1957). In the main series of carrageenin experiments thirty-six guinea pigs were used, each was injected with 50 mg. of carrageenin subcutaneously, and killed in batches of three at each interval of time. A further twenty-one guinea pigs were used for the determination of specific activities in normal rib cartilage and skin, again three guinea pigs for each interval of time. The 625 μc of ^{35}S injected was chosen to give a dosage of 1 μc/g. body wt. Any corrections necessary in individual groups of animals to bring the dosage to this level were small, but have been made throughout where required.

Isolation of polysaccharide fractions

Neutral salt-soluble polysaccharides. One-third of the total pooled granuloma tissue, macerated, was suspended in 10 times its weight of 0·2 M-NaCl (pH 7·4) at 2° and stirred occasionally during 24 hr. 2-Octanol was used as preservative. The supernatant was decanted off and extraction for a further 24 hr. was carried out. The combined supernatants were centrifuged at 1500 *g* for 15 min. and then dialysed first against running water for 24 hr. and finally against several changes of distilled water for 24 hr. The total dialysate was then brought to 1·5 M-KCl by addition of solid KCl and incubated overnight at 35° with 50 mg. of cetylpyridinium chloride in the presence of 2-octanol. By using the method of fractional precipitation of cetylpyridinium complexes (Scott, 1956) it was found that, at 1·5 M-KCl, all carrageenin present in the extract could be precipitated, and centrifuged off, with negligible loss of total ^{35}S radioactivity. Excess of cetylpyridinium chloride was removed from the supernatant by shaking for 1 hr. with 2 g. of fuller's earth. After centrifuging, the supernatant was reduced in volume to about 50 ml. by vacuum distillation on a water bath not exceeding 65°, and the polysaccharides were then precipitated on standing overnight at 2° with 3 vol. of ethanol. The polysaccharide-containing precipitate was centrifuged off, redissolved in 20 ml. of water and dialysed for 24 hr. against frequent changes of distilled water. After finally centrifuging at 1500 *g* for 15 min. the supernatant contains the neutral salt-soluble polysaccharides separated from carrageenin.

Removal of ionic sulphate by the dialysis procedure was considered to be complete since (*a*) a known amount of radioactivity added as $Na_2{}^{35}SO_4$ to the macerated tissues could be demonstrated to have been completely removed from the dialysis sac at the end of the periods stated above, and (*b*) the final dialysate water gave no trace of cloudiness on addition of barium chloride.

Polysaccharides obtained by papain digestion. After the above extraction the tissue was suspended in 100 ml. of citrate buffer, pH 6·0, with 10 mg. of dry papain powder (Griffin and Tatlock)/g. wet wt. of tissue. The papain was activated by addition of cystine and ethylenediaminetetraacetic acid to give a concentration of 0·005 M of each and incubated under 2-octanol for 24 hr. at 45°. After cooling, the insoluble debris was centrifuged off at 1500 *g* for 15 min. The residue was washed thoroughly twice with 25 ml. of water and the washing combined with the supernatant. The combined extracts were then dialysed for 24 hr. and again centrifuged. The supernatant was brought to 1·5 M-KCl with solid KCl, and the precipitation of remaining intracellular carrageenin repeated by using the cetylpyridinium chloride method described above. Polysaccharides were separated, after removal of excess of cetylpyridinium with fuller's earth, by precipitation with 3 vol. of ethanol, redissolving in 20 ml. of water, further dialysis against distilled water and final centrifuging. About the same yields of $^{35}SO_4$ were obtained from equal amounts of the same granuloma tissue when 0·6 mg./g. of wet tissue of twice-recrystallized papain (Kimmel & Smith, 1954) was substituted for the commercial papain powder.

Residual sulphate fraction. Water suspensions of the washed residual material were dialysed against distilled water for 24 hr., and as such constitute the residue sulphate fraction. Examination of this residual material under the light microscope shows it to be largely amorphous, having no particular staining characteristics. Some rough-branching fibrous material could occasionally be distinguished but it is impossible to say whether this material is elastin or not. Further digestion with fresh papain in buffer, pH 6, did not result in further dissolution of this residue material or reduce appreciably the small amount of ^{35}S activity contained in it.

Measurement of radioactivities

Samples of the three sulphate fractions above were hydrolysed for 24 hr. in 6 N-HNO_3 under reflux. After cooling, the hydrolysates were neutralized with KOH, adjusted to pH 5–6 and heated to 90° in centrifuge tubes in a water bath. To these tubes 5 ml. of 0·05 N-Na_2SO_4 and, after stirring, 3 ml. of N-$BaCl_2$ were then added and the precipitate of $BaSO_4$ was allowed to form for 1–2 hr. at 90°; the tubes were quickly centrifuged whilst hot. The $BaSO_4$ precipitate was washed first with water and finally with ethanol–ether (50:50, v/v) and dried at 110°. The radioactivity of solid disks of the $BaSO_4$ of 1 cm.^{2} surface area and 'infinite' thickness was estimated by counting with a standard thin mica end-window Geiger–Müller tube and scaling equipment.

Correction for decay time of $^{35}SO_4$ has been made throughout; no correction for self-absorption is required.

Chemical analysis

Measurement of sulphate. Sulphate determinations were done on samples of the three fractions after 24 hr. hydrolysis with 6 N-HNO_3. The hydrolysates were dried *in vacuo* in a desiccator over solid KOH. Sulphate analyses were carried out by the method of Dodgson & Spencer (1953) with the following modifications: (1) precipitation of the benzidine sulphate was on to no. 3 sintered-glass filters sealed into straight tubes (Townson and Mercer Ltd.) holding 10 ml. and fitting into centrifuge tubes. This greatly facilitates the collection of the small amount of benzidine sulphate. (2) Preliminary double washing of the precipitate is made with acetone saturated with benzidine sulphate and the final washing is with acetone alone. Reproducible results were more easily attained with these modifications, and by the use of HNO_3 rather than HCl for hydrolysis, provided that the greatest care is exercised in cleaning glassware, purifying reagents where required, and if a strictly standardized technique in the method itself is used.

Estimation of hexosamine. The method was that of Blix (1948), the reaction, however, being carried out at 90° for 1 hr. Three estimations were made on each of the three fractions from the pooled granulomata at the various time intervals after injection of carrageenin.

The absorption maximum of the colour produced from representative samples of the tissue fractions, isolated as described above, varied between 526 and 538 mμ. The readings were all made at 530 mμ.

RESULTS

After the subcutaneous injection of carrageenin there is a very rapid, and massive, growth of new connective tissue. Even by the third day, upwards of 10 g. of fresh tissue is regularly obtained, and by the fifth and sixth days amounts of 30 g. and more

are not uncommon. As will be seen from Table 1 there follows from the seventh day onwards an equally rapid resorption of the granuloma, which is eventually largely replaced by loose fatty tissue. The amounts of sulphate found in the three polysaccharide-containing fractions isolated from the granuloma are also shown in Table 1. It will be seen that the total sulphate is already at a maximum 3 days after injection of carrageenin, and falls steadily after the sixth day, reaching the levels found in the normal guinea-pig skin by the ninth to the 14th day. The amount of sulphate in the salt (0·2 M-NaCl) fraction is also highest at the third day, then falls with time, more slowly from about the seventh day. The sulphate in fraction 2, that extracted by papain digestion, is high during the whole period from the third to the seventh day and shows no definite peak. From the seventh to the 14th day there is a fall in the sulphate content of this papain fraction. On the other hand, the residue sulphate shows only small alterations in amount, being slightly higher from about the ninth day onwards.

Hexosamine determinations in the three fractions extracted from the granuloma are shown in Table 2. Total hexosamine is already high by the third day after carrageenin. It remains high until the sixth day and then falls to the amount found in normal guinea-pig skin by about the 14th day. Hexosamine in the three fractions of granuloma shows alterations with time similar to those found in sulphate from the three fractions. Hexosamine in the salt fraction 1 is already high at the third day. It then falls with time after the fifth day, at first sharply but more slowly from the seventh day onwards. In the papain fraction 2 the hexosamine content does

Table 1. *Amounts of sulphate/100 g. wet wt. of tissue in three fractions of granuloma tissue, and total wet wt. of tissue obtained, after subcutaneous injection of 50 mg. of carrageenin*

Figures are means of at least three determinations on pooled tissue from three guinea pigs at each time interval.

Time after injection of 50 mg. of carrageenin (days)	Wet wt. of tissue extracted (g.)	Salt fraction 1 0·2 M-NaCl (pH 7·4)	Papain fraction 2 10 mg. of papain/g. of tissue	Residue sulphate 3 by hydrolysis remainder	Totals 1, 2 and 3
			(mg./100 g. wet wt. of tissue)		
3	13	23	11	5	39
5	18·5	15	12	5	32
5·5	33	15	13	6	34
6	38	12	13	6	31
7	33	9	11	5	25
9	8·5	7	6	7	20
14	8·5	4	5	8	17
21	3·5	2	3	7	12
28	2·0	1	4	8	13
Normal guinea-pig skin	—	7	9	3	19

Table 2. *Amounts of hexosamine/100 g. of wet tissue in three fractions of the granuloma*

Figures are means of three or more determinations on pooled tissue from three guinea pigs at each time interval.

Time after injection of 50 mg. of carrageenin (days)	Salt fraction 1 0·2 M-NaCl (pH 7·4)	Papain fraction 2 10 mg. of papain/g. of tissue	Residue sulphate 3 by hydrolysis remainder	Totals 1, 2 and 3
		(mg./100 g. wet wt. of tissue)		
3	116	71	10	197
5	104	74	10	188
5·5	120	79	9	208
6	85	90	12	187
7	61	80	13	154
9	32	80	10	122
14	25	70	9	104
21	15	50	9	74
28	14	54	10	78
Normal guinea-pig skin	37	65	8	110

not show such large changes with time. As with the sulphate content, hexosamine in the residue fraction 3 varies little in amount throughout the time interval studied.

Table 3. *Specific activities ($^{35}SO_4$) in counts/min./0·1 mg. of sulphate found in three fractions from carrageenin-induced granuloma in guinea pigs*

$Na_2{}^{35}SO_4$, 1 μc/g. body wt. was injected intraperitoneally 5 days after 50 mg. of carrageenin had been injected subcutaneously. Radioactivities were counted with 29±0·3 mg. of carrier $BaSO_4$ as 'infinitely' thick disks 1 cm.^{2} surface area.

Time after injection of $Na_2{}^{35}SO_4$	Salt fraction 1 0·2 M-NaCl (pH 7·4)	Papain fraction 2 10 mg. of papain/g. of tissue	Residue sulphate 3 by hydrolysis remainder
2 hr.	12	14	26
6 hr.	354	23	22
8 hr.	462	44	20
12 hr.	672	128	22
24 hr.	586	327	19
2 days	438	363	13
4 days	53	245	8
9 days	22	199	12
12 days	28	143	7
16 days	10	115	6

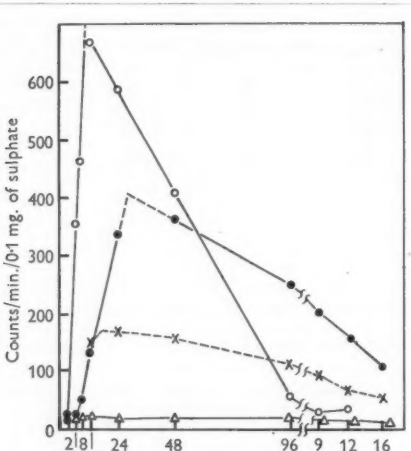

Fig. 1. Specific activity–time curves of three fractions of organically bound sulphate from carrageenin-induced granuloma in guinea pigs, and of papain-extracted polysaccharide from normal guinea-pig skin. Zero is the point of injection of 1 μc of $Na_2{}^{35}SO_4$/g. body wt. and is 5 days after injection of carrageenin. ○, Neutral-salt-fraction polysaccharide; ●, papain-fraction polysaccharide; △, residue sulphate; ×, papain-fraction polysaccharide from normal guinea-pig skin.

Specific activities of $^{35}SO_4$, in counts/min./0·1 mg. of sulphate found, in the three fractions of granuloma tissue are shown in Table 3. The highest activity was found in the salt fraction at 12 hr., but if the figures are plotted as in Fig. 1 it will be seen that the steeply rising part of the curve is bisected, at about 10 hr., when the falling part of the curve is extrapolated backwards from the 12 hr. point. Maximal activity of the salt fraction 1 sulphate is therefore probably at about 10 hr. The specific activity of the salt fraction then falls very rapidly so that it has already reached a low level by the fourth day after injection of $Na_2{}^{35}SO_4$. From the fourth to the 16th days there is a slow fall of the remaining activity in this fraction.

Maximal activity in the papain-extracted fraction was found at the 2-day point. But again the ascending part of the specific activity–time curve (see Fig. 1) was steeper than the descending portion, and if the latter is extrapolated backwards, it will be seen to bisect the ascending curve at about 36 hr. This is about the time required for maximal activity to be reached in the chondroitin sulphate of rib cartilage, see Boström (1952), Slack (1955). It will be seen from Table 3 and Fig. 1 that the maximal activity of the papain fraction is less than one-half that of the salt fraction. Furthermore, the fall in specific activity with time occurs at a slower rate than in the salt fraction.

By contrast the specific activities of the residue sulphate are low at all the time intervals studied and show relatively small fluctuations with time. The specific activity–time curve shows no definite peak and the half-life cannot be calculated on the available data.

Polysaccharides were extracted from rib cartilage and skin of normal guinea pigs after injection intraperitoneally of 1 μc of $Na_2{}^{35}SO_4$/g. body wt. After injection the animals were killed at the time intervals shown in Table 4 and portions of rib cartilage and skin, cleaned free of adjacent muscle,

Table 4. *Specific activities (counts/min./0·1 mg. of sulphate) found in normal guinea-pig rib cartilage and skin extracted with papain*

Radioactivities were counted with 29±0·3 mg. of carrier $BaSO_4$ as 'infinitely' thick disks. Three guinea pigs were taken for each time interval.

Time after injection of $Na_2{}^{35}SO_4$	Normal rib cartilage (papain fraction 2)	Normal skin (papain fraction 2)
12 hr.	84	128
1 day	138	163
2 days	150	149
4 days	124	114
9 days	109	89
12 days	94	62
16 days	79	48

fat and fascia, were extracted first with water in the cold for several hours and then digested for 24 hr. with papain, as described above under Methods. The specific activities found are shown in Table 4. The sulphate extracted by this method from both rib cartilage and skin in the normal guinea pig shows only one-half as much incorporation of $^{35}SO_4$ as the papain fraction of the carrageenin granuloma. The specific activity–time curve of the papain fraction from rib cartilage is very similar to that of normal cartilage in the rat. In the normal guinea-pig skin the peak of activity appears to be a little earlier than in the cartilage, at about 24 hr.

DISCUSSION

The granuloma produced by subcutaneous injection of small amounts of the polygalactose carrageenin has provided a most useful means of studying the active formation and breakdown of new connective tissue *in vivo*. In the preceding paper, Jackson (1957) has shown that in collagen formation there is early production of a neutral salt-soluble collagen which behaves metabolically as the precursor of formed collagen fibres.

During the early phase of the granuloma formation, in the first 5 days or so after carrageenin injection, there is a rapid build-up of polysaccharides. After the fifth or sixth day there follows a rapid fall in the polysaccharide contents, as determined by the methods described, so that from the 14th day onwards the total hexosamine and sulphate fall to about the levels found in normal guinea-pig skin. This is in general agreement with the recent experiments on wound healing in otherwise healthy rats by Dunphy & Udupa (1955). They showed that following upon wounds made in the abdominal cavity there is an early 'productive' or 'substrate' phase lasting from 1 to 5 days, during which there is a rapid formation of polysaccharides, followed by a 'collagen' phase lasting from the fifth or sixth day until healing is complete. During the 'collagen' phase they found a rapid fall in the polysaccharide content up to about the ninth day, after which the decrease occurred much more slowly. As regards total hexosamine and total sulphate in the carrageenin-induced granuloma the results are very similar. Unlike normal wound healing, however, the later stages of the carrageenin granuloma result in complete removal of the previously formed connective tissue. This is indicated by the fall in both total hexosamine and total sulphate below the values found in normal guinea-pig skin from the 21st day onwards.

When we come to consider the separate fractions of the granuloma tissue, two points stand out. First, both the hexosamine and sulphate of the residue fraction are low in amount and show little fluctuation with time. This residue fraction would appear to play little part in the dynamics of the granuloma formation or breakdown and would appear to be related to already existing elements of the subcutaneous tissue. Microscopical examination of this fraction reveals a largely amorphous debris with no characteristic staining reactions. Occasionally, however, rough branching fibrous material can be discerned which may represent pre-existing elastin (elastin is very resistant to papain digestion). The hexosamine and sulphate content of this fraction may therefore, in part at least, be in association with elastin. Secondly, the very early production of polysaccharides with a subsequent very rapid fall in both hexosamine and sulphate is most clearly seen in the salt-soluble fraction 1 of the granuloma. The build-up and breakdown of the papain fraction appears to be a somewhat steadier, more delayed process.

These differences are clearly amplified by the dynamics of the process as indicated by the specific activities found in the three fractions. The salt-soluble fraction with its high level of specific activity follows a specific activity–time curve closely similar to that of the neutral salt-soluble collagen. It may, indeed, be the soluble sulphated-polysaccharide precursor of the papain fraction, which latter is closely associated with proteins which are both water- and neutral-salt insoluble (presumably largely insoluble and acid-soluble collagen). That this possible 'precursor', neutral salt-soluble polysaccharide, itself occurs largely in association with soluble protein and not in free solution, is evident from the fact that its presence is not readily shown by paper chromatography until the protein components are denatured by heating to 70° and by ethanol precipitation. The delayed fall in specific activity of the papain fraction and its close association in solubility properties with the insoluble collagen fraction suggest its association dynamically with this collagen fraction in its formation and subsequent breakdown.

SUMMARY

1. New connective tissue, in a highly active metabolic state, was induced by subcutaneous injection of carrageenin in guinea pigs. The wet weight of the granulation tissue so induced increased rapidly to a maximum about the seventh day after carrageenin. There followed a rapid resorption of the new tissue and by the sixth week only a little fatty tissue remained at the injection site.

2. Sulphate and hexosamine determinations suggest an early, rapid synthesis of polysaccharide. This was already high on the third day, maximal about the fifth or sixth day, and then declined rapidly to reach normal levels about the 14th day.

3. $Na_2{}^{35}SO_4$ was injected intraperitoneally on the fifth day after carrageenin and animals were killed at several intervals of time afterwards. The highest specific activity found was in a neutral salt-soluble extract at 10 hr. after isotope administration. The activity of this fraction then fell rapidly with time. Sulphate extracted by papain digestion had a maximal specific activity about 30 hr. after isotope administration. Loss of activity in this fraction was less rapid than in the neutral salt-soluble extract. A residual sulphate fraction had low specific activities throughout and an indefinite half-life.

4. It is suggested that neutral salt (0·2 M-NaCl) extract of the granuloma tissue may contain a precursor sulphated polysaccharide. The very high rate of metabolism of this neutral-salt extract parallels that of neutral salt-soluble collagen and points to a close metabolic interrelationship of these two connective-tissue components.

I wish to thank Professor Kellgren for many stimulating discussions and never-failing encouragement during the course of this work, and also Drs D. S. Jackson and G. Williams for their support and collaboration throughout.

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Studies on Glucosaminidase

N-ACETYL-β-GLUCOSAMINIDASE IN RAT KIDNEY

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The occurrence of *N*-acetyl-β-glucosaminidase in mammalian tissues was described by Watanabe (1936*a*) and noted by Hahn (1945) and Roseman & Dorfman (1951). Watanabe (1936*b*) and East, Madinaveitia & Todd (1941) studied the properties of the enzyme in partially purified preparations from ox liver and testis.

Hahn (1945) and East *et al.* (1941) showed that *N*-acetyl-β-glucosaminidase and hyaluronidase, which are present together in crude testicular extracts, are different enzymes. Linker, Meyer & Weissmann (1955) have shown that the oligosaccharides formed from hyaluronic acid by hyaluronidase ('poly-*N*-acetyl-β-glucosaminidase') are further degraded stepwise by β-glucuronidase and *N*-acetyl-β-glucosaminidase. In view of the probable importance of *N*-acetyl-β-glucosaminidase in the biochemistry of mucopolysaccharides and in studies on the structure of derivatives of *N*-acetylglucosamine, further work on the preparation and properties of this enzyme seemed desirable. A preliminary survey of rat tissues showed that kidney was the most active source of the enzyme.

The kinetics of hydrolysis of phenyl *N*-acetyl-β-D-glucosaminide by a partially purified preparation of *N*-acetyl-β-glucosaminidase from rat kidney have been investigated and a study has been made of some factors which influence the activity of the enzyme.

METHODS

Preparation of substrate. Phenyl *N*-acetyl-β-D-glucosaminide was prepared by a modification of the method of Helferich & Iloff (1933).

Preparation of the enzyme. Fresh rat kidney was ground for 1 min. with water in a stainless-steel homogenizer. The homogenate (2%, w/v) was brought to pH 4·8 with citrate buffer (final concn. 0·1 M) and centrifuged for 15 min. at 1500 *g*. The supernatant was fractionated at 0–4° by addition of saturated ammonium sulphate. The fraction precipitated between 20 and 50% saturation was dissolved in water and dialysed overnight at 0–4°. After centrifuging to remove inactive, insoluble material, the preparation was diluted to the original volume of homogenate to give an enzyme concentration suitable for assay by the method described below. Such preparations contained about 70–80% of the activity of the homogenate. The activity fell about 10% on storage for 1 week at 0–4°.

Estimation of enzyme activity

This was carried out by a modification of the method of East *et al.* (1941). Tubes containing substrate (0·01 M), buffer (0·05 M citrate, pH 4·3) and enzyme (0·1 ml.) in a final volume of 1·0 ml. were incubated at 37° for 1 hr. Each

estimation was carried out in duplicate. Control tubes, in which enzyme and substrate respectively were omitted, were also incubated. The selection of these standard conditions for the estimation was based on the results of the experiments described below.

Estimation of liberated phenol. At the end of the incubation period, the reaction was stopped by the addition of 2·0 ml. of Folin–Ciocalteu reagent (British Drug Houses Ltd., diluted 1 in 3). After centrifuging to remove precipitated protein, 2·0 ml. of the supernatant was added to 3·0 ml. of Na_2CO_3 (12%, w/v). Colour was developed by incubation at 37° for 20 min. and the absorption at 650 mμ. was measured in a Unicam SP. 500 spectrophotometer. After correcting for the enzyme and substrate blanks, which were very low, the amount of phenol liberated by the enzyme was read from a calibration curve constructed with a standard solution of phenol. Any additional component included in the reaction mixture was tested to see that it did not affect the colour reaction for phenol. The enzyme concentration was such that the amount of substrate hydrolysed did not exceed 5% of that initially present.

Estimation of liberated N-acetylglucosamine. The addition of 0·1 ml. of 3N-Na_2CO_3 at the end of the incubation period brought the pH to 10·8 and stopped the enzyme reaction. The estimation of *N*-acetylglucosamine was completed as described by Aminoff, Morgan & Watkins (1952). The absorption was read at 560 mμ. and the amount of *N*-acetylglucosamine liberated by the enzyme was estimated from a standard curve made from a solution of *N*-acetylglucosamine in 0·05M citrate buffer, pH 4·3. The colour in the control tubes was very low and it was shown that the colour reaction for *N*-acetylglucosamine was not affected by the presence of either substrate or enzyme solution.

RESULTS

Nature of the enzyme reaction

The molar ratio of phenol to *N*-acetylglucosamine liberated on incubation of phenyl *N*-acetyl-β-glucosaminide with *N*-acetyl-β-glucosaminidase under the standard conditions described above was 0·99 ± 0·02 (mean ± S.E.) in three experiments, each carried out with a different preparation of the partially purified enzyme. Other experiments showed that under the same conditions neither phenol nor *N*-acetylglucosamine underwent any destruction on incubation with the enzyme. These results provide strong support for the assumption that the reaction involved is in fact a simple hydrolysis of the substrate.

Effect of pH

The effect of pH on the rate of hydrolysis of phenyl *N*-acetyl-β-glucosaminide by the enzyme was determined over the range pH 3–6·5 in 0·05M citrate buffer at a substrate concentration of 0·01M. Incubation and phenol estimation were carried out as described above. The results are shown in Fig. 1. A smooth curve with a single pH optimum at 4·3 was obtained. Watanabe (1936*b*) reported a pH optimum of about 4 for the enzyme in ox liver.

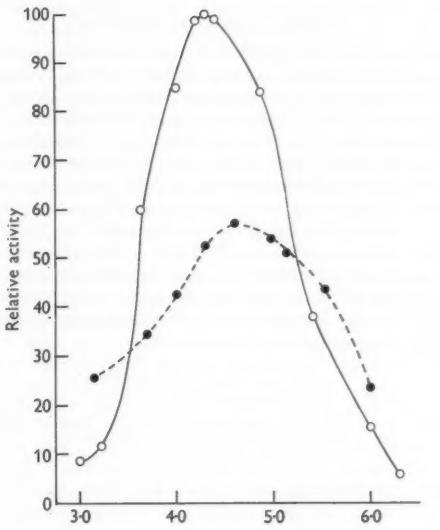

Fig. 1. Effect of pH on reaction velocity. Incubation in 0·05M citrate or acetate buffer for 1 hr. at 37°. Substrate: 0·01M phenyl *N*-acetyl-β-glucosaminide. ○, Citrate buffer; ●, acetate buffer.

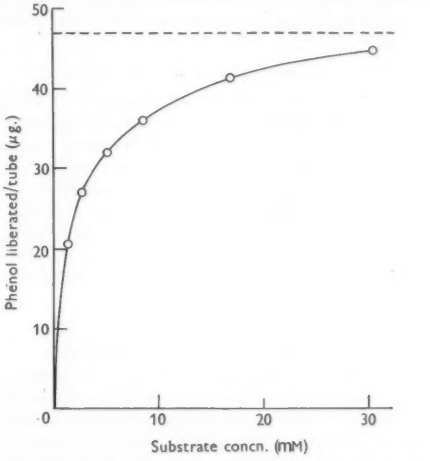

Fig. 2. Effect of varying substrate concentration on reaction velocity. The enzyme solution was incubated for 1 hr. at 37° in 0·05M citrate buffer, pH 4·3, with varying concentrations of phenyl *N*-acetyl-β-glucosaminide. Final vol. of reaction mixture was 1·0 ml. containing 0·1 ml. of enzyme solution. ○—○, Plot of reaction velocity against substrate concn.; – – – –, theoretical maximum velocity derived as described in the text.

Effect of substrate concentration

Fig. 2 shows the effect of varying substrate concentration on the reaction velocity in 0·05 M citrate buffer, pH 4·3. The rate of hydrolysis increased with increase in the substrate concentration, giving a typical Michaelis–Menten curve. A true maximum in the rate of hydrolysis was not obtained even at the highest substrate concentration used, but it is not possible to extend the curve further since the reaction mixture is almost saturated with substrate at a concentration of 0·03 M. The theoretical maximum velocity of hydrolysis at infinite substrate concentration, shown in Fig. 2, was found by plotting the results according to the equation derived by Lineweaver & Burk (1934):

$$1/v = 1/V_{max.} + K_m/V_{max.}S,$$

where S is the substrate concentration, v and $V_{max.}$ the observed and maximum velocity respectively and K_m the Michaelis constant (cf. Fig. 5). The mean value for K_m found in ten experiments was 1·78 mM phenyl *N*-acetyl-β-glucosaminide (Table 3), which is of the same order as the value of 3 mM found by Watanabe (1936*b*) for the enzyme in ox liver.

A substrate concentration close to the optimum cannot be conveniently employed in routine estimations owing to the limited solubility of the substrate. The value 0·01 M which was selected is arbitrary but, as can be seen from Fig. 2, it is high enough on the curve to ensure that substrate concentration is not a critical variable in the results obtained.

Effect of enzyme concentration

Fig. 3 shows that under standard conditions for the estimation of *N*-acetyl-β-glucosaminidase activity the amount of phenol liberated was proportional to the amount of enzyme present over an eightfold range of enzyme concentration. The amount of substrate hydrolysed by the highest concentration of enzyme was only 15% of that initially present and there was no indication of a decline in the rate of hydrolysis due to inhibition by reaction products.

Effect of time of incubation

The effect of varying the period of incubation on the hydrolysis of phenyl *N*-acetyl-β-glucosaminide, under conditions which were otherwise standard, is shown in Fig. 4. The rate of liberation of phenol was proportional to the time of incubation up to 1 hr. but after this it declined progressively. It is probable that this fall is due to instability of the enzyme on prolonged incubation, even in the presence of substrate, and not to either substrate exhaustion or inhibition by reaction products (cf. Fig. 3). In contrast to these results Watanabe (1936*b*) found that a partially purified enzyme preparation from ox liver showed no loss of activity on incubation for 10 hr. even though 87% of the substrate initially present was split.

Stability of the enzyme

The partially purified preparation of *N*-acetyl-glucosaminidase from rat kidney was comparatively stable to dialysis and subsequent storage at 0–5° (see above). At 37° in 0·05 M citrate buffer, pH 4·3, however, the enzyme was unstable in the absence of

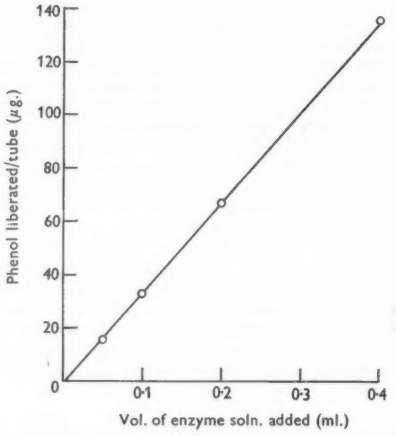

Fig. 3. Effect of enzyme concentration on reaction velocity. Varying amounts of enzyme solution were incubated in 0·05 M citrate buffer, pH 4·3, for 1 hr. at 37°. Substrate: 0·01 M phenyl *N*-acetyl-β-glucosaminide; final vol. of reaction mixture was 1·0 ml.

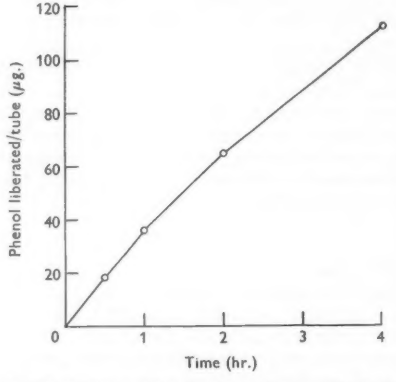

Fig. 4. Effect of time of incubation on degree of hydrolysis. The reaction was allowed to proceed for varying periods at 37° in 0·05 M citrate buffer, pH 4·3. The substrate was 0·01 M phenyl *N*-acetyl-β-glucosaminide.

substrate, the activity falling to 50 % of its initial value in about 90 min. (Table 1). The stability of the enzyme in acetate buffer of the same molarity and pH was much greater. This difference in the effect of the two buffers on the stability of the enzyme was also observed with crude tissue homogenates and is almost certainly due to combination between the enzyme and acetate, which results in stabilization of the enzyme in the absence of substrate and inhibition of its activity in the presence of substrate (see below).

Effect of various substances on the activity of N-acetyl-β-glucosaminidase

The following substances, present in the assay mixture at a final concentration of 0·01 M, had no effect on the enzyme activity: NaCl, KCl, $MgCl_2$, NaF, KCN, ethylenediaminetetraacetic acid, glucosamine hydrochloride.

Activity in acetate and other buffers

Acetate, which was the buffer employed by East *et al.* (1941) and Hahn (1945) in their work on β-glucosaminidase, was also used in the early stages of this work. In acetate buffer the pH optimum of the enzyme from rat kidney was 4·6 (Fig. 1), in agreement with the value found by East *et al.* (1941) for a partially purified preparation from ox testis. The substrate concentration curve in acetate buffer was abnormal. Enzyme activity increased irregularly with increasing substrate concentration and showed no sign of flattening off even at the highest attainable substrate concentration. It was then found that the activity of the enzyme was considerably higher in citrate buffer than in equimolar acetate of the same pH, and that in citrate the activity was independent of the molarity of the buffer, whereas in acetate activity fell progressively with increasing acetate concentration (Table 2). The effect of other buffers, at two different molarities, on the activity of the enzyme was also tested. The results (Table 2) show that at a final concentration of 0·1 M the activity in formate, propionate, lactate and succinate was 80–100 % of the activity in citrate. A fourfold reduction in the molarity of the buffer resulted in a 10–15 % fall in the activity of the enzyme in each case.

Inhibition by acetate, acetamide and N-acetylglucosamine

It seemed possible that the inhibition of the enzyme produced by acetate might be competitive. Fig. 5 shows the results of an experiment in which the effect of varying substrate concentration on the activity in citrate buffer was tested in the presence and absence of a fixed concentration of acetate, the final pH being the same in both cases. The results, plotted according to Lineweaver & Burk (1934), show that acetate behaves as a typical competitive inhibitor.

Table 1. *Stability of N-acetyl-β-glucosaminidase at pH 4·3 and 37°*

Activity was measured after pre-incubation in 0·05 M buffer solution for varying periods. Results are expressed as percentage residual activity.

Period of pre-incubation (hr.)	Residual activity (%)	
	Citrate buffer	Acetate buffer
1	59	91
2	39	88
4	33	87

Table 2. *Effect of nature of buffer and concentration of buffer on N-acetyl-β-glucosaminidase activity*

Incubation lasted 1 hr. at 37°, pH 4·3. The substrate was 0·01 M phenyl *N*-acetyl-β-glucosaminide. Results are expressed as percentage of the activity in 0·1 M citrate buffer.

Buffer	Activity (%)			
	0·1 M	0·05 M	0·025 M	0·01 M
Citrate	100	100	99	95
Acetate	44	51	59	65
Formate	97	—	85	—
Propionate	82	—	75	—
Succinate	99	—	83	—
Lactate	91	—	78	—

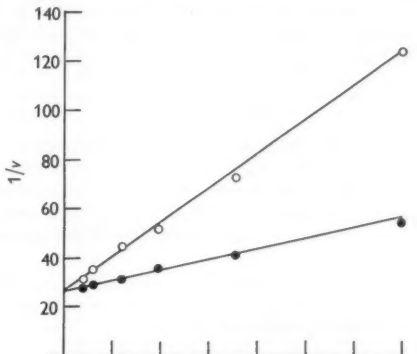

Fig. 5. Effect of varying substrate concentration on the rate of hydrolysis of phenyl *N*-acetyl-β-glucosaminide in the presence (○), and the absence (●), of 0·045 M acetate buffer, pH 4·3. Incubated for 1 hr. at 37° in 0·05 M citrate buffer, pH 4·3. Results are plotted according to Lineweaver & Burk (1934); *s*, 10(substrate concn.) (M); *v*, mg. of phenol liberated/tube.

Watanabe (1936*b*) showed that *N*-acetyl-β-glucosaminidase was inhibited by its reaction product, *N*-acetylglucosamine. This has been confirmed in the present work and it was found that the inhibition was competitive, as might be expected. Since acetate and *N*-acetylglucosamine had been found to inhibit competitively, it seemed likely, on structural grounds, that acetamide would behave similarly, and this was confirmed by experiment. Table 3 summarizes the results obtained. The values of K_m and K_i, which are assumed to measure the dissociation constants of the enzyme–substrate and enzyme–inhibitor complexes, were evaluated graphically according to Lineweaver & Burk (1934). The figures for the apparent relative affinities of the enzyme for the substrate and for the three inhibitors are derived from the reciprocals of the dissociation constants.

In addition to the theoretical assumptions implicit in the derivation of these figures, there are other considerations which have been left out of account. The mutarotation of *N*-acetylglucosamine, which is present in solution as a mixture of α- and β-forms, has been studied only in water at 20° (Kuhn & Haber, 1953). At pH 4·3 and 37° no difference between the inhibitory powers of a freshly prepared and an aged solution of *N*-acetylglucosamine could be demonstrated, even in experiments with an incubation period as short as 10 min. Nevertheless, it is probable that each of the anomers has a different affinity for the enzyme. With acetate the direction and amount of the change in the pH optimum from pH 4·3 in citrate to 4·6 in acetate is of the order predicted from the assumption that inhibition is due to un-ionized acetic acid and not to the acetate ion.

DISCUSSION

The results described above have extended the early work of Watanabe (1936*a*, *b*) on the properties of *N*-acetyl-β-glucosaminidase in mammalian tissues. The partially purified enzyme preparation from rat kidney showed no evidence of the presence of more than one active component. It was stable to dialysis, showed no activation or inhibition by common ions and in citrate buffer the kinetics of hydrolysis of phenyl *N*-acetylglucosaminide by the enzyme were simple.

Table 3. *Apparent affinity of inhibitors for* N*-acetyl-β-glucosaminidase compared with that of phenyl* N*-acetyl-β-glucosaminide*

Compound	Apparent dissociation constant* (mM)	Apparent relative affinity
Phenyl *N*-acetyl-β-glucosaminide	1·78±0·10 (10)	100
N-Acetylglucosamine	4·40±0·66 (3)	41
Acetamide	10·2±0·35 (3)	18
Acetate (pH 4·3)	17·0±2·2 (3)	11

* Mean±S.E. and, in parentheses, number of experiments.

In testicular extracts, β-glucosaminidase has been distinguished from β-glucuronidase (Hahn, 1945), hyaluronidase (Hahn, 1945; East *et al.* 1941) and α-glucosaminidase (Roseman & Dorfman, 1951), and from β-glucosidase in emulsin (Helferich & Iloff, 1933) and in extracts of snail digestive tract (Neuberger & Pitt Rivers, 1939). The specificity of the mammalian enzyme has not been studied. With the snail enzyme, Neuberger & Pitt Rivers (1939) showed that the requirements for hydrolysis were that the substrate should be a β-glucosaminide with an *N*-acetyl or *N*-formyl group on $C_{(2)}$ and with the hydroxyl groups on $C_{(3)}$, $C_{(4)}$ and $C_{(6)}$ unsubstituted. The inhibition of *N*-acetyl-β-glucosaminidase by acetate has not hitherto been described. We have confirmed that this inhibition can also be shown in a testicular enzyme preparation similar to that used by East *et al.* (1941). Although the results of Neuberger & Pitt Rivers (1939) do not suggest any inhibition by acetate of the snail enzyme, it is interesting to note that Roseman & Dorfman (1951) found that the activity of *N*-acetyl-α-glucosaminidase was greater in phosphate–citrate buffer than in acetate. Veibel & Lillelund (1940) observed that the rate of hydrolysis of *o*-cresol glucoside by β-glucosidase was about 10% less in acetate than in citrate buffer. However, a normal type of substrate-concentration curve was obtained in acetate and the lower activity was explained by the relatively greater affinity of enzyme for its reaction products in this buffer. Although inhibition due to such a mechanism would probably appear to be competitive in the type of analysis carried out by us, it seems much more likely that the marked inhibition of *N*-acetyl-β-glucosaminidase by acetate is due to a direct interaction of the enzyme with acetate to form an inactive complex. The increasing apparent relative affinity of the enzyme for acetate, acetamide and *N*-acetylglucosamine supports this interpretation, as does the greater stability of the enzyme incubated in the absence of substrate in acetate as compared with citrate buffer.

The conditions selected for the assay of the enzyme in partially purified preparations have been found suitable for use with unfractionated tissue homogenates, and further work on the specificity and distribution of the enzyme is being carried out.

SUMMARY

1. The hydrolysis of phenyl *N*-acetyl-β-D-glucosaminide by *N*-acetyl-β-glucosaminidase in a partially purified extract of rat kidney has been

investigated, and conditions for the estimation of the enzyme have been established.

2. In 0·05 M citrate buffer, the enzyme shows optimum activity at pH 4·3. The optimum substrate concentration cannot be attained owing to the limited solubility of the substrate.

3. *N*-Acetyl-β-glucosaminidase is inhibited competitively by *N*-acetylglucosamine, acetamide and acetate.

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The Action of 2:4-Dinitrophenol on Myosin and Mitochondrial Adenosine Triphosphatase Systems

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Mitochondria freshly isolated from liver show low or negligible adenosine triphosphatase (ATPase) activity towards added substrate, but on addition of low concentrations of 2:4-dinitrophenol (DNP), adenosine triphosphate (ATP) is hydrolysed at a rapid rate (Hunter, 1951; cf. Lardy, 1945). Similar, though perhaps less striking, effects have been obtained with mitochondria isolated from skeletal muscle (Chappell & Perry, 1954). The precise nature of this effect of DNP is not understood, although, significantly, it is accompanied by the loss of oxidative phosphorylating activity (Loomis & Lipmann, 1948; Cross, Taggart, Covo & Green, 1949) and by the inability of ATP to reverse the swelling induced in mitochondria of pigeon breast muscle by hypotonic media (Chappell & Perry, 1954). In isolated mitochondria the action of DNP is complex and may involve, amongst other things, effects on the permeability of the membrane; but it appears from the work of Lardy & Wellman (1953) on the ATPase activity of aqueous extracts of mitochondrial acetone-dried powders that DNP has a specific stimulatory effect on the ATPase enzyme itself. Furthermore, the observation that DNP stimulates the ATPase activity of myosin (Webster, 1953) suggests that DNP can have an effect on the mechanism of ATP hydrolysis when it is catalysed by other enzymic systems.

In previous studies on the metabolism of ATP by intracellular components of muscle (Chappell, 1954; Chappell & Perry, 1953, 1954) we found that under the ionic conditions in which DNP stimulated the enzymic activity of L-myosin (Weber & Portzehl, 1952), 0·1–5 mM DNP failed to stimulate the myofibrillar ATPase activity of rabbit myofibrils. The present communication is concerned with a general investigation of the effects of DNP on the ATPase activity both of myosin and isolated myofibrils and shows that the presence of actin modifies the action of DNP on the enzymic activity of myosin. The response of the latter system to DNP has been compared with that of the mitochondrial ATPase.

Certain aspects of this work have already been reported (Chappell & Perry, 1955). In an associated communication, Greville & Needham (1955) reported similar findings on the DNP stimulation of L-myosin ATPase activity.

METHODS

Myofibrils. Myofibrils were prepared in a medium containing 0·039 M borate buffer, pH 7·1, and 0·025 M-KCl (borate–KCl) as previously described (Perry & Grey, 1956). Stock preparations were stored at 0°, and for enzyme experiments they were used within 10–12 days, diluted with borate–KCl to give suspensions containing 5–7 mg. of protein/ml.

Myofibrillar proteins. L-Myosin was prepared by a method described elsewhere (Perry, 1955) which combines the experience of a number of workers. The method of Guba & Szent-Györgyi (1945) was used for the preparation of G-actin solutions. G-Actin was purified by isoelectric

precipitation with 0·02 M sodium acetate buffer, pH 4·8. The concentrated F-actin solution (10–20 mg. of protein/ml.) which was obtained on restoring the pH to 7 was stored at 0°.

Mitochondria and mitochondrial extracts. Rat-liver mitochondria were prepared in 0·25 M sucrose as described by Schneider (1948), and pigeon breast-muscle mitochondria by the method of Chappell & Perry (1954). The method of Lardy & Wellman (1953) was employed for the preparation of acetone-dried powders of rat-liver mitochondria. To obtain extracts these powders were homogenized at 0° with 20 vol. of glass-distilled water in a pestle-type homogenizer, allowed to stand 30 min. at 0° and centrifuged for 20 min. at 1000 *g*. The supernatant was used for ATPase experiments.

ATPase experiments. Enzyme experiments were carried out in general as described earlier (Perry & Grey, 1956), at 0° or 20°. The reagents were pre-incubated for 5–15 min. at the incubation temperature and the reaction was started by addition of 0·2 ml. of enzyme suspension. Unless otherwise stated, the ATP (sodium salt, obtained from Sigma Chemical Co., St Louis, U.S.A.) was used at a concentration of 0·005 M. The pH values of buffer solutions were in all cases measured at 20°.

2:4-*Dinitrophenol.* This was twice recrystallized either from ethanol or water.

RESULTS

Effect of DNP on the calcium-activated ATPase of myofibrils and L-*myosin.* The addition of 1–10 mM DNP to the standard incubation medium produced a progressive inhibition of the calcium-activated ATPase (Ca-ATPase) of myofibrils prepared in borate–KCl. In the presence of 0·05 M-KCl, a similar range of DNP concentrations produced little change in ATPase activity, but as the ionic strength of the medium was increased still further a marked stimulation of the Ca-ATPase activity by DNP was obtained (Fig. 1*a*). Although the addition of the higher concentrations of KCl decreased the ATPase activity in the absence of DNP, the maximum

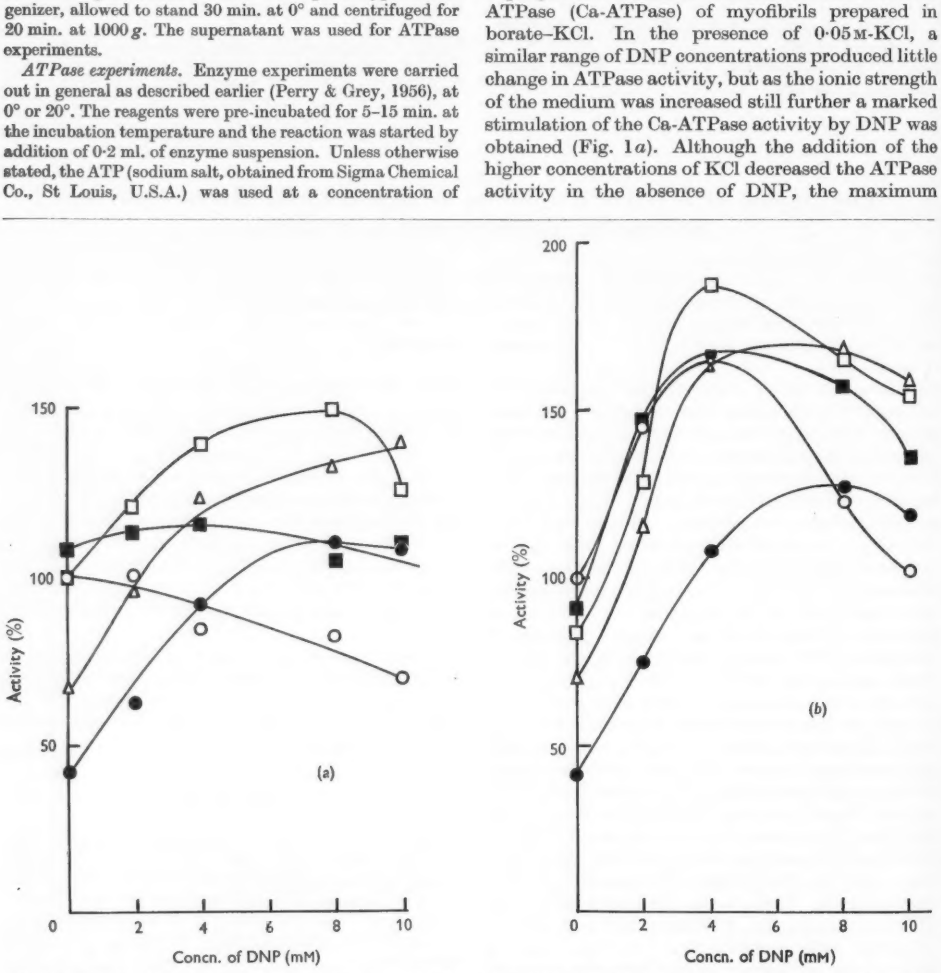

Fig. 1. Effect of DNP on the Ca-ATPase of (*a*) myofibrils (3 days old) and (*b*) L-myosin, at varying ionic strengths. Incubations were carried out for 10 min. at 20° in a total volume of 2 ml. containing 5 mM ATP, 5 mM-$CaCl_2$, 0·05 M tris buffer (pH 7·4), 0·1 ml. of myofibrils in borate–KCl or myosin (about 0·06 mg. of N), in 0·04 M-KCl, and the following further additions: ○, nil; ■, 0·05 M-KCl; □, 0·1 M-KCl; △, 0·2 M-KCl; ●, 0·4 M-KCl. Activity in the absence of added DNP and KCl was taken as 100.

stimulation obtained in the presence of the phenol was greater than the depression of activity produced by salt alone.

The Ca-ATPase of L-myosin was also markedly stimulated by concentrations of DNP similar to those used in the experiments with myofibrils (Fig. 1*b*). A major difference existed in the response of myosin to DNP, however, in that stimulation was achieved in the absence of added KCl, i.e. at the ionic strength of the incubation medium (approx. 0·06).

The effect of KCl on the Ca-ATPase of the myofibrillar system was not a specific one but rather an effect of ionic strength, for NaCl was equally effective and for a given DNP concentration K_2SO_4 produced maximal stimulation at a lower concentration.

In the myofibril the myosin is associated with actin and it seemed probable that the presence of the latter protein was responsible for the difference in response to DNP of the ATPases of the two systems. The fact that another property of myosin which is modified by actin, i.e. magnesium activation of the ATPase, is likewise sensitive to ionic strength over a similar range, supported this view. To check this hypothesis, synthetic actomyosins containing a fixed amount of myosin and varying amounts of actin were prepared and the effect of DNP on their ATPase activities in the standard incubation medium (*I* approx. 0·06) was determined. The results of one such experiment are illustrated in Fig. 2 and indicate that when the ratio myosin N : actin N was 2, DNP no longer stimulated the ATPase activity. They also show that the stimulating effect of DNP is restored by the addition of 0·2 M-KCl to this actomyosin system.

Effect of DNP on the magnesium-activated myofibrillar ATPase. When magnesium was used to activate the myofibrillar ATPase (Mg-ATPase), an increase in the ionic strength progressively decreased the activation effect, which was entirely absent when the incubation medium contained 0·2 M-KCl. Nevertheless, with freshly prepared myofibrils, magnesium appreciably stimulated the ATPase in the presence of 0·1 M-KCl (see Perry & Grey, 1956). Addition of DNP to this system at concentrations of 0·1 and 1 mM had little or no effect on the Mg-ATPase; 5 mM DNP inhibited at all levels of ionic strength (Fig. 3). This is in contrast with the effect on Ca-ATPase, where, under similar ionic conditions, stimulation by DNP increased progressively with concentration up to a maximum at 8 mM.

Effect of DNP on manganese-activated myofibrillar ATPase. Activation of the myofibrillar ATPase by $MnCl_2$ resembled the effects obtained with $MgCl_2$ in that with increasing ionic strength the activation fell off sharply until, in the presence of 0·2 M-KCl, the ATPase activity was at a low level. The addition of increasing amounts of DNP, in the presence of KCl up to 0·05 M, produced inhibition or activation,

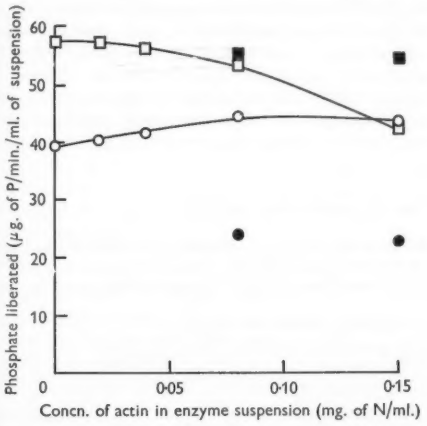

Fig. 2. Effect of actin on the stimulation of the Ca-ATPase of myosin by DNP. Incubation medium contained 0·05 M tris buffer (pH 7·4), 5 mM-$CaCl_2$, 5 mM ATP and 0·2 ml. of enzyme suspension in 0·05 M-KCl; final volume, 2 ml. The enzyme solutions contained a fixed amount of myosin (0·28 mg. of N/ml.). ○, No further additions; □, 5 mM DNP; ●, 0·2 M-KCl; ■, 0·2 M-KCl + 5 mM DNP.

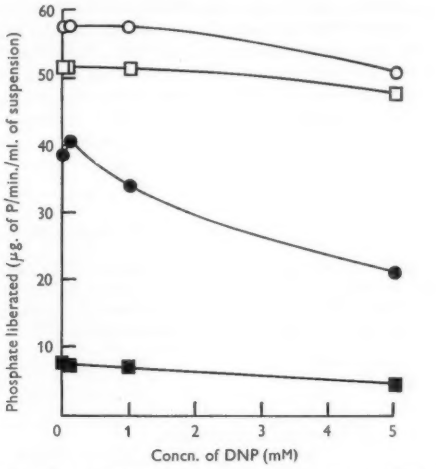

Fig. 3. Effect of DNP on the Mg-ATPase of myofibrils at varying KCl concentrations. Myofibrils, 1 day old. Incubations were carried out for 5 min. at 20° with 5 mM ATP, 5 mM-$MgCl_2$, 0·05 M tris buffer (pH 7·4), and the following further additions: ○, nil; □, 0·05 M-KCl; ●, 0·1 M-KCl; ■, 0·2 M-KCl.

neither of which was very pronounced. At higher KCl concentrations the ATPase activity was lower, but increasing amounts of DNP produced stimulation. Thus, although at higher ionic strengths greater ATPase activity was obtained with calcium than with manganese, the systems were similar in that activity in both cases was stimulated by DNP.

Reversibility of DNP effect. A number of experiments were performed which demonstrated that after the myofibrillar Ca-ATPase had been stimulated by DNP under typical conditions, and the phenol largely removed by dialysis, restimulation occurred on addition of fresh DNP. Samples of both control and DNP-treated myofibrils were dialysed against the borate–KCl medium and then assayed for the DNP effect in the presence and absence of 0·2M-KCl. The results of a typical experiment are presented in Table 1.

An attempt was made to assay the amount of ATPase brought into solution under the conditions used to study stimulation of the Ca-ATPase by DNP. This was done by cooling to 0° a sample of the complete incubation mixture after incubation with and without DNP, and then centrifuging at this temperature for 5 min. at 20 000 *g*. The supernatant, which was considered to contain any soluble ATPase, was then assayed again at 20° in a medium identical with the original. In the absence of added KCl no Ca-ATPase activity passed into solution, but with added KCl (0·2M) most of the ATPase-active material activity was rendered soluble. In general the results indicated that, under conditions in which DNP stimulated the myofibrillar ATPase, the enzyme was partly in solution. Nevertheless, DNP did not invariably stimulate when the enzyme was in solution, for although 60–70% of the ATPase-active material was soluble in the presence of 0·2M-KCl at 0°, DNP produced inhibition under these conditions.

Effect of DNP on calcium-activated ATPase at 0°. When the effect of DNP on the Ca-ATPase of L-myosin and myofibrils was compared at 0° rather than at 20° it was noted that many of the differences observed in the behaviour of the two systems at the higher temperature had disappeared. For example, at 0° in the absence of added KCl, increasing DNP concentration caused progressive inhibition of the Ca-ATPase of L-myosin, and stimulation was not obtained until the concn. of KCl was 0·4M (Fig. 4*b*). Similar results were obtained with the Ca-ATPase of the myofibrils (Fig. 4*a*), and apart from the fact that in the absence of DNP increasing ionic strength caused a greater increase in enzymic activity with myosin than with myofibrils, the sets of curves obtained were almost identical.

It was noted that on cooling to 0° the standard incubation medium containing 0·05M aminotris-hydroxymethylmethane (tris) buffer, pH 7·4, at 20°, the pH rose by approximately 0·4. This change in pH of the medium on cooling, however, was not responsible for the different behaviour of DNP at 0°, for the following reasons. At 20° no significant difference could be detected in the behaviour of L-myosin to DNP in 0·05M tris, pH 7·4 or pH 7·8. Likewise the behaviour of L-myosin in 0·05M glyoxaline buffer, pH 7·0, to DNP at 0° and 20° was very similar to that observed under similar conditions in 0·05M tris, pH 7·4.

Magnesium activation of the myofibrillar ATPase at 0°. The result obtained on reducing the temperature from 20° to 0° for the DNP stimulation of the Ca-ATPase of myosin prompted the study of certain other properties of the enzyme at the lower temperature. In particular it was noted that at 0° magnesium inhibited the myofibrillar ATPase at approximately mM concentration and activated only very slightly, if at all, in the range 5–10 mM. This meant that over this range the Mg-ATPase activity

Table 1. *Reversal of effect of DNP on the myofibrillar ATPase*

The myofibrils (11 days old) were assayed for ATPase under the conditions indicated, dialysed, and assayed again with the additions shown. The enzyme incubation medium contained 0·05M tris buffer (pH 7·3), 5 mM-$CaCl_2$ and 5 mM ATP in all cases. Dialysis was carried out against KCl–borate for 16 hr. at 0° with three changes. Results are expressed as μg. of P liberated/min./ml. of myofibril suspension at 20°.

Additions to assay medium, before dialysis	ATPase activity after dialysis: No added KCl – DNP	No added KCl + DNP	No added KCl % inhibition by DNP	0·2M-KCl – DNP	0·2M-KCl + DNP	0·2M-KCl % stimulation by DNP
None	37·1	30·1	19	24·2	44·1	82
5 mM DNP (produced 13% inhibition)	41·5	30·8	26	23·7	47·6	101
0·2M-KCl	41·0	30·9	25	22·7	48·5	103
5 mM DNP + M-KCl (produced 220% stimulation)	45·2	36·0	20	26·1	52·4	101

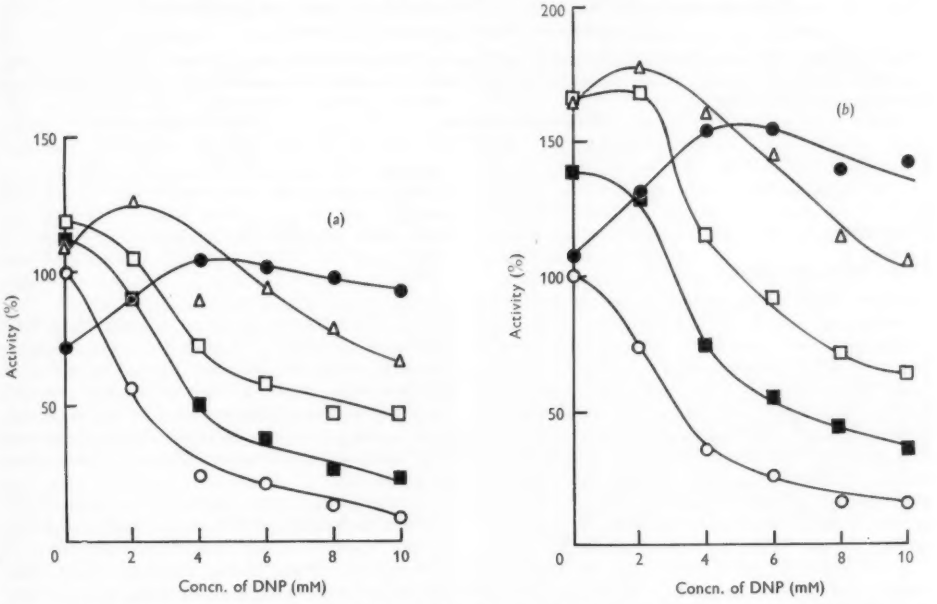

Fig. 4. Effect of DNP on the Ca-ATPase of (*a*) myofibrils (7 days old) and (*b*) L-myosin, at varying ionic strengths. Incubations were carried out for 60 min. at 0°. Conditions otherwise the same as those of Fig. 1. Activity in absence of DNP and KCl was taken as 100. ○, No added KCl; ■, 0·1 M-KCl; □, 0·2 M-KCl; △, 0·4 M-KCl; ●, 0·8 M-KCl.

fell by a factor of 50 or 60 on reducing the temperature from 20° to 0°, whereas, in contrast, the Ca-ATPase activity of myofibrils was decreased by a factor of 5 or 6 and the pattern of activation remained unchanged. The results of these experiments are summarized in Fig. 5, as are experiments carried out at pH 7·8 which clearly indicate that the effect on the characteristics of magnesium activation is due to temperature and not to the pH shift obtained on cooling the buffer (see previous section).

Mitochondrial ATPase

Whole mitochondria. It is well known (Kielley & Kielley, 1951) that freshly prepared liver mitochondria hydrolyse ATP very slowly, but that on the addition of 0·1 mM DNP the ATPase activity rises to a high value (the so-called latent ATPase). This effect is obtained with DNP approximately 10–50 times more dilute than that required to produce stimulation of the ATPase of myosin or myofibrils, but there appear to be fundamental similarities in the two systems. To throw light on these the effect of temperature on the DNP stimulation of the mitochondrial ATPase was investigated.

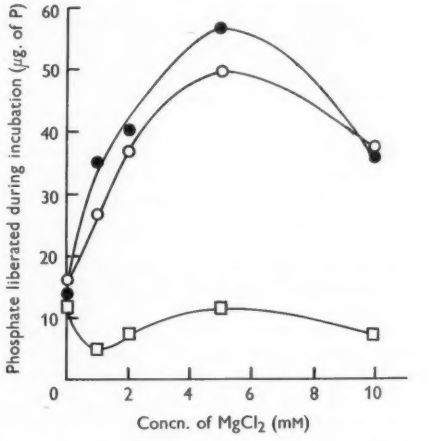

Fig. 5. Activation of the myofibrillar ATPase by magnesium at 0° and 20°. Incubation was carried out in 0·05 M tris buffer, 5 mM ATP. Myofibrils, 3 days old. ○, Incubated at 20° for 5 min. at pH 7·4; ●, incubated at 20° for 5 min. at pH 7·8; □, incubated at 0° for 30 min. at pH 7·4.

Table 2. *Action of DNP on the ATPase activity of fresh liver mitochondria and aqueous extracts of acetone-dried powders*

The incubation medium contained 0·05 M tris buffer, 5 mM ATP, and in the experiments with whole mitochondria, 0·1 M-KCl to maintain tonicity. All pH values were measured at 20° and results are expressed as μg. of P liberated/min. by 1 ml. of enzyme preparation.

	Whole mitochondria			Acetone-dried powder		
	pH 7·3		pH 7·8	pH 7·4		pH 7·8
Additions	20°	0°	20°	20°	0°	20°
Nil	1·8	0·2	4·6	4·6	0·6	3·9
0·1 mM DNP	160	2·4	130	5·8	0·5	5·9
5 mM-$MgCl_2$	12	1·2	18	18·2	0·9	22·5
0·1 mM DNP + 5 mM-$MgCl_2$	146	3·4	142	25·3	0·9	32·4

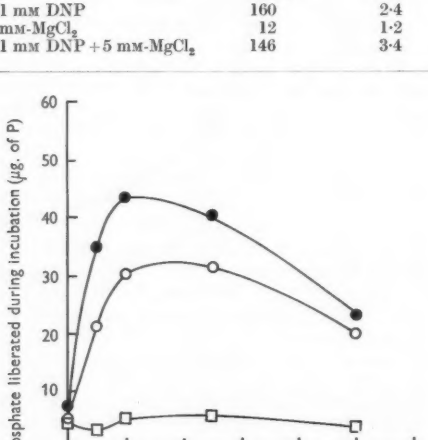

Fig. 6. Activation of ATPase of an aqueous extract of liver mitochondrial acetone-dried powder. Conditions the same as those of Fig. 5. ○, Incubated at 20° for 5 min. at pH 7·4; ●, incubated at 20° for 5 min. at pH 7·8; ■, incubated at 0° for 30 min. at pH 7·4.

Table 2 shows that at 0° the DNP stimulation of the ATPase of whole rat-liver mitochondria is very much reduced. This effect was obtained in the absence or presence of $MgCl_2$ in the incubation medium. After pre-incubation with 0·4 mM DNP for 30 min. at 0° the mitochondria were activated on subsequent incubation at 20° in a manner identical with control mitochondria not pre-incubated. Similar effects were obtained with pigeon breast-muscle mitochondria, although it should be noted that in general these mitochondria have appreciable ATPase activity when freshly prepared and in consequence the extent of stimulation by DNP is not so great (Chappell & Perry, 1954).

If the mitochondria were pre-incubated with 0·4 mM DNP under the conditions of the enzyme test, i.e. approximately isotonic, at 0° or 20°, the DNP could be removed by centrifuging and washing with isotonic solutions in the cold without activation of the ATPase. Subsequent addition of DNP produced reactivation of such DNP-treated mitochondria, although in some cases the activated ATPase level attained was not so high as that obtained with control mitochondria which had not been initially treated with DNP. This suggests that DNP can cause some irreversible inactivation of the enzyme.

Acetone-dried powder. In view of the complexity of the mitochondrial structure compared with that of the myofibril and the possible importance of permeability effects in the study of the ATPase activity of the former, direct comparison of the ATPase activity of these two systems is difficult. A more satisfactory mitochondrial ATPase system for this purpose is the aqueous extract of the acetone-dried powder of mitochondria which has been reported by Lardy & Wellman (1953) to possess ATPase activity stimulated by DNP.

Whereas it was not necessary to add $MgCl_2$ to whole liver mitochondria to obtain full activity in the presence of DNP, with the acetone-dried powder extract $MgCl_2$ was essential for full activity (Table 2). As with the myofibril, the mitochondrial ATPase was not activated by magnesium at 0° (Table 2 and Fig. 6), indicating that a high temperature coefficient between 0° and 20° is characteristic of both magnesium-activated ATPases. Also, as might be expected, DNP did not activate the mitochondrial Mg-ATPase at 0° (Table 2). This suggests that the lack of effect of DNP on the ATPase of whole mitochondria at 0° may be in part due to the fact that magnesium does not activate at this temperature. Likewise manganese was found to produce little activation at 0°, whereas at 20° it was at least as effective as magnesium in this respect.

DISCUSSION

The evidence presented indicates that at low ionic strength actin interacts with myosin in a manner which prevents DNP from producing its stimulating effect on the myosin ATPase; in fact, at very low ionic strength DNP is inhibitory to the actomyosin-

ATPase system. The mechanism of DNP stimulation of ATP hydrolysis is obscure, but in view of the effects obtained in the presence of actin it is reasonable to presume that this protein can in some way modify the nature or sequence of events at the enzymically active centres of the myosin. The view that the mechanism of ATP hydrolysis may be modified by actin is also supported by the well-known fact that at low ionic strength magnesium activates the ATPase activity of actomyosin but not of myosin (Banga & Szent-Györgyi, 1943). It is tempting to suppose that there are two slightly different mechanisms for the hydrolysis of ATP by myosin systems, namely one that involves calcium only and another in which either calcium or magnesium can participate. Both mechanisms have many common features, but whereas the former is typical of myosin over a wide range of ionic strength and of actomyosin at ionic strength above 0·15–0·20, the latter occurs in the presence of actin at ionic strengths lower than 0·15–0·20. It is suggested that only the former is stimulated by DNP. Bearing pertinently on this point is the finding that DNP begins to stimulate the ATPase of myofibrils at just that range of ionic strength at which magnesium activation disappears.

It is not clear what changes are induced in the actomyosin system when the ionic strength rises above 0·15–0·20, but certainly the activation properties characteristic of actomyosin disappear and those of myosin remain. It is unlikely that complete dissociation of the actomyosin takes place, but probably there occurs some 'loosening' of the interaction which modifies the state of affairs in the vicinity of the active centres of the myosin so that the influence of the actin moiety is lost.

It is important to decide whether the action of DNP on myosin ATPase presents a model for the study of the action of this phenol on the hydrolysis of ATP by isolated mitochondria, in view of the possible relation of the latter system to oxidative phosphorylation. The extent of stimulation of the ATPase of intact liver mitochondria by DNP is very much greater than that obtained with myosin or myofibrils. It is very questionable, however, if the two systems are strictly comparable, since the extremely low ATPase activity of fresh intact liver mitochondria may be due to the impermeability of the mitochondrial membrane to ATP (see Siekevitz & Potter, 1955). Certainly with isolated pigeon breast-muscle mitochondria, which have high ATPase activity when freshly prepared, and with aqueous extracts of acetone-dried powders of liver mitochondria, the extent of activation is much more comparable to that obtained with the myosin system.

A difference exists in that optimum activation of the myosin ATPase system is obtained with DNP concentrations 10–50 times as great as those effective on the mitochondrial ATPase. This fact might imply a difference in mechanism of action, but alternatively it may be the result of a difference in affinity of the two systems for DNP. In systems splitting ATP at comparable rates there would be much less mitochondrial ATPase protein than myosin because of the relatively low specific activity of the latter. It is possible also that the relative affinity of the two systems for DNP is determined by the high lipid content of the mitochondrial preparations.

Another point of difference is that the Mg-ATPase of mitochondria is stimulated by DNP, whereas with myosin the Ca-ATPase is the one which is stimulated. Despite this fact the magnesium-activated systems of both mitochondria and myosin are similar in that magnesium activates both systems little or not at all at 0°. Hasselbach (1952) and Gilmour & Calaby (1952) have also commented on the high temperature coefficient of magnesium-activated actomyosin and insect-muscle apyrase respectively. It would be of interest to investigate the effect of temperature on other enzyme systems requiring magnesium and ATP to determine whether this observation is a general one for such systems and reflects perhaps certain features of the complexing action of magnesium for ATP.

SUMMARY

1. In confirmation of Webster's (1953) finding, 2:4-dinitrophenol has been shown to stimulate the calcium-activated adenosine triphosphatase (ATPase) of myosin. The stimulation amounted to 100–150% under optimum conditions, and at 20° occurred when the ionic strength was varied over the range of approx. 0·06–0·46.

2. When actin was present in a medium of low ionic strength, 2:4-dinitrophenol inhibited the calcium-activated ATPase of myosin. 2:4-Dinitrophenol stimulated the calcium-activated ATPase of myofibrils at ionic strength of about 0·16 and higher.

3. At 0° the ionic strength had to be raised to about 0·4 and higher to obtain stimulation of the calcium-activated ATPase activity of L-myosin and myofibrils by 2:4-dinitrophenol.

4. The pattern of calcium activation of the myofibrillar ATPase was similar at 0° and 20°. In contrast, whereas magnesium strongly activated at 20°, at 0° it produced little or no activation of rabbit-myofibrillar or rat-liver mitochondrial ATPases.

5. Some effects of the action of 2:4-dinitrophenol on the rabbit-myofibrillar and the rat-liver mitochondrial ATPases have been compared and discussed.

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Ultraviolet Fluorescence of the Aromatic Amino Acids

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Compounds resembling the aromatic amino acids are known to show appreciable fluorescence in the near ultraviolet [Ley & Englehardt (1910), Kowalski (1911), Marsh (1924)], but the fluorescence of the aromatic amino acids themselves has not so far been unequivocally characterized. Debye & Edwards (1952) have made observations of the phosphorescence of the aromatic amino acids. The position of the phosphorescence bands and the decay times found by these authors indicate the probable existence of fluorescence bands in the near ultraviolet with normal decay times (McClure, 1949).

It is shown in this paper that the three aromatic amino acids exhibit characteristic ultraviolet fluorescence. The fluorescence-excitation spectra, fluorescence spectra and quantum yields of aqueous solutions have been studied. Further papers will deal with aspects of the ultraviolet fluorescence of peptides and proteins.

Characterization of a substance as a fluorescent entity

It is often necessary to determine whether the fluorescence shown by a solution is due to a given substance present in it. To ascribe the observed fluorescence to a given component of the system the following criteria are proposed:

Fluorescence-excitation spectrum. The quantum yield of the fluorescence of a substance in *solution*, defined as the ratio of the number of quanta emitted to the number of quanta absorbed, is known to be independent of the exciting wavelength, at least for excitation with light in the air ultraviolet ($\lambda > 2000$Å) and visible regions of the spectrum (Wavilov, 1927; Neporent, 1947; Weber & Teale, in preparation). Therefore,

$$F(\lambda) = kqA(\lambda), \tag{1}$$

in which $F(\lambda)$ is the fluorescence intensity set up by excitation with light of wavelength λ, $A(\lambda)$ the number of photons absorbed in the solution, q the quantum yield and k a constant depending on the general geometry of the system and the distribution of the intensity along the exciting beam. A solution of optical density $E(\lambda)$ illuminated with light of this wavelength of intensity $I(\lambda)$ photons absorbs

$$A(\lambda) = I(\lambda)\,(1 - 10^{-E(\lambda)}). \tag{2}$$

From (1) and (2)

$$F(\lambda) = qkI(\lambda)\,(1 - 10^{-E(\lambda)}). \tag{3}$$

For an arbitrary wavelength $\bar{\lambda}$

$$qk = F(\bar{\lambda})/I(\bar{\lambda})\,(1-10^{-E(\bar{\lambda})}). \qquad (4)$$

If (4) is used to eliminate qk from (3)

$$F(\lambda) = \frac{F(\bar{\lambda})\,I(\lambda)}{I(\bar{\lambda})}\left[\frac{1-10^{-E(\lambda)}}{1-10^{-E(\bar{\lambda})}}\right].$$

$\log \Omega$ may now be defined as

$$\log \Omega \equiv \log 1 \Big/ \left\{1-\frac{F(\lambda)/F(\bar{\lambda})}{I(\lambda)/I(\bar{\lambda})}\,(1-10^{-E(\bar{\lambda})})\right\} = E(\lambda). \qquad (5)$$

$\bar{\lambda}$ may be conveniently chosen to be the wavelength of the maximum of the absorption band of least frequency. Then $F(\lambda)\,I(\bar{\lambda})/F(\bar{\lambda})\,I(\lambda)$ is simply the fluorescence relative to that set up by excitation with the wavelength of the absorption maximum referred to equal photon excitation. Equation (5) shows that a plot of $\log \Omega$ against λ should reproduce the absorption spectrum of the fluorescence entity which may be identified in this way. It must be stressed that k is very sensitive to a change in the distribution of the fluorescent intensity along the exciting beam, and therefore equation (5) is only applicable when $E(\lambda)$ is small. In practice $E(\lambda)$ must be less than 0·1, preferably less than 0·05. When $E(\bar{\lambda})$ may be reduced to 0·05 or less equation (5) may be reduced to the simpler form

$$E(\bar{\lambda})\,\frac{F(\lambda)\,I(\bar{\lambda})}{F(\bar{\lambda})\,I(\lambda)} = E(\lambda). \qquad (6)$$

Fluorescence spectrum. It is known that the fluorescence spectrum continues the absorption spectrum towards longer wavelengths. Although the difference between the maximum of absorption λ_A and the maximum of emission λ_F varies considerably from one substance to another and often in the same substance depending on the solvent, always $\lambda_F > \lambda_A$. This provides a useful criterion to exclude certain components of a solution as responsible for the observed fluorescence.

Quantum yield. The possibility that the fluorescence of a solution may be due to an impurity can often be resolved by a knowledge of the overall quantum yield. In a system consisting of several components the overall quantum yield due to excitation by light of wavelength λ is

$$\overline{q(\lambda)} = q_a\,\frac{E_a}{E(\lambda)} + q_b\,\frac{E_b}{E(\lambda)} + \ldots + q_n\,\frac{E_n}{E(\lambda)},$$

where q_a, q_b, ..., are the quantum yields and $E_a/E(\lambda)$, $E_b/E(\lambda)$, ..., the fractional absorptions of the respective components. Let the principal component of the system be a. Since all q's $\leqslant 1$ and

$$E(\lambda) = E_a + E_b + \ldots + E_n,$$

$$\overline{q(\lambda)} \leqslant q_a\,\frac{E_a}{E(\lambda)} + \frac{E(\lambda)-E_a}{E(\lambda)};$$

but also $E_a/E\lambda \leqslant 1$. Therefore

$$\left.\begin{aligned} \overline{q(\lambda)} &\leqslant q_a + \left[1-\frac{E_a}{E(\lambda)}\right] \\ \text{and} \quad q_a &\geqslant \overline{q(\lambda)} - \left[1-\frac{E_a}{E(\lambda)}\right]. \end{aligned}\right\} \qquad (7)$$

Thus in stable substances purified by crystallization a quantum yield of a few per cent indicates that the fluorescence is due to the main component of the system.

EXPERIMENTAL

The fluorescence-excitation spectra were determined by means of the apparatus the block diagram of which is shown in Fig. 1. Light from a high-pressure Nester-type hydrogen arc H enters the monochromator after passing through the collimating system K. The monochromatic beam emerges from the exit slit X into the light-tight box B containing the cell C filled with the fluorescent solution. The cell axis makes an angle of 15° with the line of view of the detector so that reflected exciting light is thrown on to the blackened wall of the box opposite to the detector. This is a 27M3 Mazda photomultiplier. The envelope of this type of photomultiplier is transparent to wavelengths longer than 2200 Å. A stabilized power supply was used to energize the photomultiplier (Fellgett, 1954) and the photoelectric currents were read directly on a Scalamp galvanometer. The stability of the system permitted the determination of photoelectric currents to within $\pm 2{\cdot}10^{-3}$ μA. In solutions of amino acids the scattering of the exciting light was negligible and no filter was required to separate the fluorescent from the exciting light. Ordinary fused-quartz cells such as those used in ordinary spectrophotometric work were found to give a blue fluorescence when irradiated with light of wavelength 230–260 mμ. The response of the photomultiplier was thus a response to the fluorescence of both cell and solution. Although a correction for the cell fluorescence is simply done by determining the response to the cell and solvent on one hand and to the cell and solution on the other, it is important to keep this correction as small as possible. The fluorescence of cells made of Ultrasil Haereus

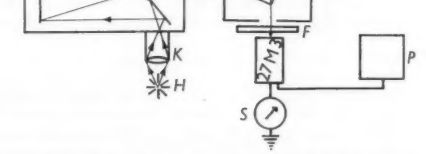

Fig. 1. Experimental arrangement for determination of excitation spectra of fluorescence. H, Nester-type hydrogen arc; K, collimating system; M, grating monochromator; X, exit slit; B, light-tight box containing the silica cell C with the fluorescence solution; F, filter to eliminate exciting light; 27M3, Mazda photomultiplier with P, power pack and S, Scalamp galvanometer.

(Quartz Glass Ltd., Barkingside, Essex) was found to be six to eight times weaker than that of cells of ordinary fused quartz. Such cells were used throughout this work. The monochromator was a Bausch & Lomb instrument with an aluminium grating blazed for the first order in the ultraviolet, giving a dispersion of 3·3 mμ./mm. at the exit slit. To determine excitation spectra the relative distribution of energy in the spectrum of the source was obtained by two methods. The first was the blackening of a photographic plate (e.g. Weissberger, 1946), the validity of the reciprocity law being assumed. The second method consisted in the use of a concentrated solution of a fluorescent substance as a proportional photon counter (Bowen, 1936; Bowen & Sawtell, 1937). As the fluorescent yield and spectrum are independent of the exciting wavelength, if the concentration of the solution is such that total absorption of the beam takes place in a very thin layer at the front of the cell, the dimensions of the fluorescent volume, and therefore the geometry of the system, remain approximately independent of wavelength and the response of the detector will be proportional to the number of photons absorbed by the solution. At the concentrations required for complete absorption of the light in a very thin layer ($>10^{-2}$ M) many fluorescent substances form an appreciable proportion of non-fluorescent dimers. As the absorption spectrum of the dimers differs from that of the monomers the fluorescent yield will be found to vary with wavelength. The selection of a suitable fluorescent substance for this purpose was therefore a matter of trial and error. Substances in which the fluorescent yield of the concentrated solution increases markedly with temperature or in which the absorption spectrum of the concentrated solutions shows absorption bands absent in the dilute solutions are therefore inadequate for this purpose (Förster, 1951). If water solutions were used, a relatively small molecular ion offered the best chances of remaining unaggregated at high concentrations. Solutions (10^{-2} M) of sodium 1-dimethylaminonaphthalene-5-sulphonate, 1-dimethylaminonaphthalene-7-sulphonate and 1-aminonaphthalene-3:6:8-trisulphonate in water were used. These substances have the further advantages that the overlap of the absorption and emission bands is negligible and that the quantum yields of the first two are very high (0·75 for 1-dimethylaminonaphthalene-7-sulphonate, 0·53 for the 1:5 isomer) (Weber & Teale, 1956).

Fig. 2 shows that the results obtained with these salts agreed very satisfactorily with the photographic method. Finally, if the direct response of the photomultiplier to the source was corrected, with the spectral-response curve provided by the manufacturers, good agreement with the other methods was obtained for the wavelengths to which the photomultiplier envelope was transparent ($\lambda > 2200$ Å).

Fluorescence spectra

These were determined with the monochromator as shown in Fig. 3. The cell *C* containing the fluorescent solution was mounted in front of the collimator *K* of the monochromator. The fluorescence was excited by light from the low-pressure mercury arc (Westinghouse Sterilamp 794) filtered through a 3 cm. layer of 0·01 M *p*-nitrophenol and M-$NiSO_4$ solution in water. This combination effectively isolated the 2537 Å line, which carried over 90% of the unfiltered energy output of the source from the other ultraviolet mercury lines. When very weak ultraviolet fluorescences were studied with this excitation filter the weak mercury lines at 2980, 3130 and 3350 Å were recorded and appeared superimposed on the fluorescent spectrum. Fortunately with the aromatic amino acids the fluorescence was sufficiently strong to make unnecessary any correction for the breakthrough of these lines. As shown in Fig. 3 the fluorescent light leaves the cell after traversing a variable thickness of solution where some of the fluorescent light may be reabsorbed to excite further fluorescence. The spectrum of the fluorescent light entering the monochromator is the technical spectrum (Birks, 1954) which differs from the molecular spectrum in two respects: the emission at wavelengths at which the absorption and fluorescence spectra overlap appreciably appears attenuated, and since the fluorescence spectrum is independent of the exciting wavelength, the re-emission of part of the absorbed light increases the emission at those wavelengths where overlap is negligible. Thus the corrections to be applied to obtain the molecular from the technical spectrum are an attenuation correction and a re-emission correction.

Attenuation correction. If $\alpha(\lambda)$ is the transmission coefficient of the solution for wavelength λ, $\bar{l}$ the average thickness of solution traversed by the rays entering the

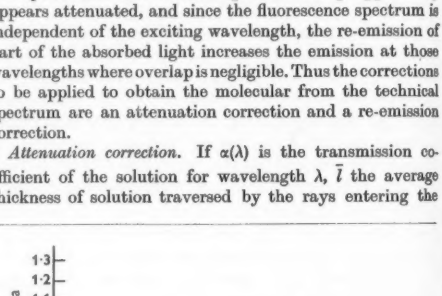

Fig. 2. Spectral photon distribution of the hydrogen arc, as emerging from grating monochromator. The continuous line is the plot of the distribution obtained by the use of fluorescent solutions of naphthylaminesulphonic acids as photon counters. The crosses are the values from the Ilford ultraviolet Q3 plates, using the reciprocity law.

Fig. 3. Experimental arrangement for determination of fluorescence spectra. Hg, Low-pressure mercury arc (Sterilamp 974); *Q*, quartz lens; *L*, liquid filter (3 cm. layer of 0·01 M *p*-nitrophenol and M-$NiSO_4$); *C*, cell containing fluorescent solution; *K*, *M*, *P*, *S* and *X* as in Fig. 1.

monochromator and $F(\lambda)$ the intensity at the exit slit corrected for the change of response of the detector with wavelength and for losses in the grating, then $F(\lambda)/\alpha(\lambda)^2$ is the intensity corrected for attenuation.

Re-emission correction. Although it is difficult to derive an exact expression for the re-emission correction, the order of this correction may be estimated as follows. The ratio of the areas under the emission bands with and without correction for attenuation is

$$\beta = \int F(\lambda)/\alpha(\lambda)^2 \, d\lambda \Big/ \int F(\lambda) \, d\lambda. \qquad (8)$$

The fractional contribution of the secondary fluorescence to the total fluorescence entering the monochromator is $(\beta - 1)\,q$, and this will be the fractional distortion of the band when the correction is ignored. If a sufficiently dilute solution is used $\beta - 1$ can be reduced to a few per cent. In such cases if q itself is small the re-emission correction may be altogether ignored as it falls within the experimental error of the determinations.

Correction for attenuation by the grating. The relative attenuation by the grating was determined as follows: light emerging at the exit slit of the grating monochromator was allowed to fall upon the entrance slit of a D. 246 Hilger prism monochromator, the detector being placed at the exit slit of the latter instrument. The band width of the light emerging from the grating instrument was kept large so that the effective band width of the light reaching the detector was always determined by the prism instrument. The source was then placed directly in front of the entrance slit of the prism instrument and the response of the detector to the range of wavelengths again determined. The ratio of the response to light reaching the detector through grating and prism to the response through prism alone is proportional to the grating transmission for this wavelength. The slit widths of both instruments were kept constant throughout the calibration. The relative attenuation by the grating was found to change slowly with wavelength, less than 0·5%/mμ. in the region of most rapid change. Thus the distortion introduced by ignoring the grating correction is small for the relatively narrow fluorescent bands but becomes important in the determination of relative quantum yields, when the areas under the bands emitted at very different wavelengths have to be compared.

Quantum yields of fluorescence

The method of Weber & Teale (1956) was used to determine the quantum yield of the fluorescence of the amino acids. In this method the intensity of the fluorescence emitted at right angles to the direction of excitation is compared with the intensity of the light scattered in the same direction by a glycogen solution. If the exciting radiation is monochromatic and the apparent optical densities of the scattering and fluorescent solutions are the same, the glycogen solution acts as a standard of quantum yield 1. Then the ratio of the fluorescent to the scattered intensity is proportional to the quantum yield, after a correction for the unequal spatial distribution of the radiation has been applied. This correction may be computed from measurements of the polarization of the radiation, and a detailed analysis leads to the equation:

$$q = \frac{S_F(3 + p_F)\, f(\lambda_0)}{S_S(3 + p_s)\, \overline{f(\Delta\lambda)}}. \qquad (9)$$

In this equation S_S and S_F are the slopes in the plot of the signal from the scattering and fluorescent solutions respectively against the optical density E when E tends to zero. p_s and p_F are the linear polarizations of the right-angle scattering and fluorescence respectively; $f(\lambda_0)$ and $\overline{f(\Delta\lambda)}$ are factors characterizing the response of the detector to a photon of scattered exciting light of wavelength λ_0 and the average response to a photon of fluorescent light respectively. Thus

$$\overline{f(\Delta\lambda)} = \int_{\Delta\lambda} f(\lambda)\, I(\lambda)\, d\lambda \Big/ \int_{\Delta\lambda} I(\lambda)\, d\lambda. \qquad (10$$

In the last equation $I(\lambda)$ is the intensity of the fluorescence emitted at wavelength λ. From a knowledge of the fluorescence spectrum and the spectral response of the photomultiplier, $\overline{f(\Delta\lambda)}$ may be easily computed by graphical integration. On the other hand if a solution of a fluorescent substance is used as a proportional photon counter, $f(\lambda_0)/\overline{f(\Delta\lambda)} = 1$. Both methods have been used in the present case. A 10^{-2} M solution of 1-dimethylaminonaphthalene-5-sulphonate was used as a proportional photon counter as already described. Glycogen solutions were used to scatter the 2537 Å radiation. The polarizations of the scattering and fluorescence were measured by means of a polarizer of fourteen quartz coverslips mounted at the Brewster angle. The experimental values were corrected for transmission of the unwanted component which was approximately 4% (Conn & Eaton, 1954). Relative quantum yields may be determined from the areas under the emission bands. If $E(\lambda)$ is the energy received by the detector from an interval of wavelength $\Delta\lambda$, $E(\lambda)/\Delta\lambda$ is the energy emitted in unit wavelength interval about λ. If $n(\lambda)$ is the number of photons in the same interval,

$$\frac{E(\lambda)}{\Delta\lambda} = n(\lambda)\,\frac{hc}{\lambda}, \qquad (11)$$

with $F(\lambda)$ denoting the signal from the detector corrected for change in response with wavelength and for losses in the monochromator, the quantity $\lambda F(\lambda)$ is proportional to $n(\lambda)$ if $\Delta\lambda$ is kept constant throughout. The areas under the plot of $\lambda F(\lambda)$ against λ are proportional to the number of photons emitted and the ratio of two such areas obtained under *identical conditions of excitation* is the ratio of the quantum yields. The condition of constant $\Delta\lambda$ is simply fulfilled with a grating monochromator by keeping the slit width constant, and this is the reason for the choice of λ rather than ν in the present instance. The optical densities were measured by means of a Uvispek spectrophotometer. Of the substances used the amino acids were commercial samples several times recrystallized. The dimethylaminonaphthalene derivatives were prepared according to Fussgänger (1902); 1-aminonaphthalene-3:6:8-sulphonate was a technical product recrystallized three times from 10% NaCl solution.

Precision and errors

The fluorescence-excitation spectra and fluorescence spectra could be reproduced to ± 1%, i.e. the area under the bands of two samples done under similar conditions would differ by 1% on the average.

The precision of the quantum-yield determination is discussed elsewhere. In general, the reproducibility gives $\Delta q = \pm 0{\cdot}01$ for $q < 0{\cdot}20$ and $\Delta q = \pm 0{\cdot}02$ for $0{\cdot}20 < q < 0{\cdot}5$.

RESULTS

Fluorescence-excitation spectra

Figs. 4–6 show the fluorescence-excitation spectra of the three aromatic amino acids plotted with the corresponding absorption spectra. Close correspondence was obtained for all three substances, including the fine structural details in phenylalanine.

Fluorescence spectra

They are shown in Fig. 7. In tryptophan and phenylalanine the overlap of emission and absorption bands is negligible and $F(\lambda)$ has been plotted as giving directly the molecular spectrum. In tyrosine, where appreciable overlap of the bands occurs, for a 10^{-5} M solution in a cell of 5 mm. thickness, $(\beta-1)\simeq 0{\cdot}1$ and $q(\beta-1)\simeq 0{\cdot}02$. The values have been corrected for attenuation only and $F(\lambda)/\alpha(\lambda)\bar{l}$ has been plotted as the molecular spectrum. Table 1 gives the position of the maxima of emission and the half-width of the bands. From an inspection of the values it appears that in a mixture of these amino acids it will be possible in many cases to detect the contribution from each of them.

In contrast to the absorption spectrum and the fluorescence-excitation spectrum, the emission spectrum of phenylalanine gives no sign of the presence of fine structure, although the resolution of the monochromator and the band width used in the scanning of the spectrum would certainly be

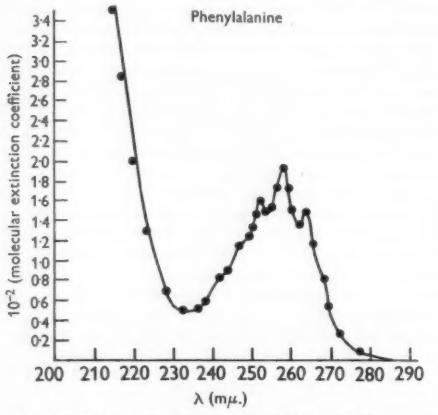

Fig. 4. Excitation spectrum of phenylalanine fluorescence in water. Abscissa: wavelength (mμ.). Ordinate: molecular extinction coefficient. The continuous line is the optical density spectrum; the dots are the values of log Ω in equation (5).

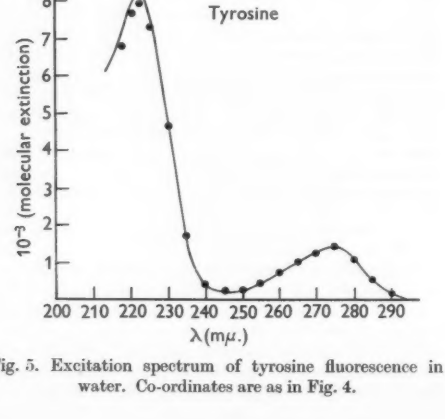

Fig. 5. Excitation spectrum of tyrosine fluorescence in water. Co-ordinates are as in Fig. 4.

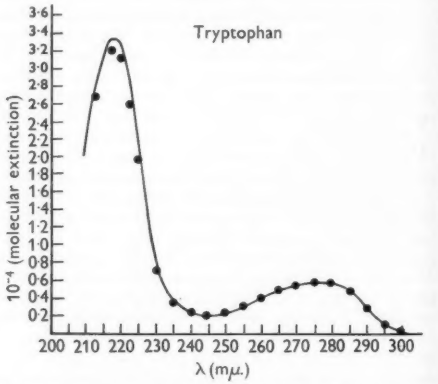

Fig. 6. Excitation spectrum of tryptophan fluorescence in water. Co-ordinates are as in Fig. 4.

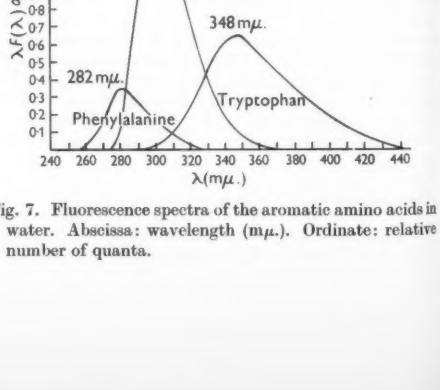

Fig. 7. Fluorescence spectra of the aromatic amino acids in water. Abscissa: wavelength (mμ.). Ordinate: relative number of quanta.

Table 1. *Maxima and band widths of the fluorescence of the aromatic amino acids in neutral aqueous solution*

λ_M is the emission maximum, $\lambda_{\frac{1}{2}}$ and $\lambda_{-\frac{1}{2}}$ are the two values of λ at which the emission becomes one-half of that at the maximum. All values are in mμ.

	λ_M	$\lambda_{\frac{1}{2}}$	$\lambda_{-\frac{1}{2}}$
Phenylalanine	282	298	270
Tyrosine	303	321	287
Tryptophan	348	383	323

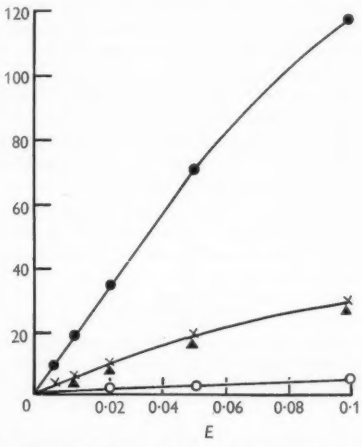

Fig. 8. Quantum yield of the aromatic amino acids in water. Integrating screen: 10^{-2} M solution of sodium 1-dimethylaminonaphthalene-5-sulphonate. Abscissa: optical density of solutions. Ordinate: signal from photomultiplier. ●, Glycogen; ×, tyrosine; ▲, tryptophan; ○, phenylalanine.

Table 2. *Quantum yield of neutral aqueous solutions of the aromatic amino acids*

q_1 is the quantum yield obtained by the use of a concentrated solution of 1-dimethylaminonaphthalene-5-sulphonate as an integrating screen, q_2 is the quantum yield obtained by using the direct response of the photomultiplier and equation 10. The value of $\overline{f(\Delta\lambda)}/f(\lambda_0)$ calculated from the fluorescence spectra and the change of detector response with wavelength is given in the third column. q_r is the yield relative to tyrosine as measured by the area under the emission band corrected for grating transmission. The relative grating transmission factors g calculated are shown in the last column.

	q_1	q_2	$\overline{f(\Delta\lambda)}/f(\lambda_0)$	q_r	g
Phenylalanine	0·045	0·038	0·97	0·23	0·95
Tyrosine	0·21	0·21	0·93	1	0·99
Tryptophan	0·19	0·20	0·76	0·92	0·887

capable of revealing it. This disappearance of fine structure in the fluorescence spectrum indicates the increased interaction between the excited molecular oscillator and the permanent water dipoles nearby. The fine structure of the phenylalanine emission spectrum may conceivably be found in polypeptides and proteins where the water dipoles in the vicinity of the amino acid are replaced by other entities. Fig. 7 also shows that approximately 15% of the quanta from tryptophan are emitted at wavelengths longer than 395 mμ., appearing as a violet fluorescence to the unaided eye.

Quantum yields

Fig. 8 shows the signal from the detector plotted against the optical density E of the solutions at 2537 Å, for solutions of the three amino acids in water (pH 6·5–7) and for glycogen solutions. The polarizations of the fluorescence for the three amino acids in water are less than 1/100, whereas the polarization of the 2537 Å radiation scattered from glycogens was found to be $0{\cdot}85 \pm 0{\cdot}01$. Therefore $3+p_F/3+p_s = 0{\cdot}78$ for the three amino acids. Most of the depolarization of the scattered light from glycogen is probably due to divergence of the exciting beam, which was uncollimated. The values of q calculated are shown in Table 2.

The *relative* efficiencies of the aromatic amino acids have also been measured by integration of the bands obtained in plotting the energy response of the detector against wavelength for constant wavelength band width as shown in Fig. 7. The relative values obtained by integration are in agreement with the absolute values obtained by the previous method, as shown in Table 2.

DISCUSSION

The preceding results show that the fluorescence observed in solutions of the aromatic amino acids is due to the amino acids themselves and not to accidental impurities present in the solutions.

In a recent publication of Shore & Pardee (1956) data are presented on the ultraviolet fluorescence of the aromatic amino acids which are at variance with those presented in this paper. The absolute fluorescence efficiencies quoted by these authors are two to three times lower than those given in Table 1. The relative efficiencies are also different, tyrosine being given as having only two-thirds of the tryptophan fluorescence. Moreover, a variation of the fluorescent efficiency with exciting wavelength, of up to 100% in one case, is claimed. We believe that these results are due to the method employed by Shore & Pardee for the determination of the fluorescent efficiencies. Their method depends on the comparison of the response of the photomultiplier to the exciting light on one hand and to

the fluorescence set up by the absorption of a known fraction of the exciting light on the other, the fluorescent and exciting light being separated by filters. Thus corrections for the geometry, integrated transmission of the filter and integrated response of the photomultiplier have to be calculated for each fluorescent substance. Although by methods neither fully described nor referred to in the paper the first two corrections have been estimated to one significant figure and the third calculated from technical data to the same accuracy, the authors give some of their results to three significant figures. Although the absolute values obtained under these conditions can be taken only as a very rough approximation, their relative figures are explained by the use of a filter with a cut-off at 300 mμ. to separate excitation from emission. Fig. 7 shows that such a filter will intercept about 80% of the phenylalanine fluorescence, 40% of the tyrosine and a negligible fraction of the tryptophan fluorescence. An overall transmission factor of 0·6 was used by Shore & Pardee for the three amino acids. The change in fluorescent efficiency with wavelength must be attributed to systematic errors in the method used, since the much more accurate technique of the excitation spectra gives no indication of such changes.

The overlap of the emission spectrum of tyrosine with its own absorption band is of the order of that found in fluorescein and rhodamine B, where transfer of the electronic energy from an excited molecule to a nearby molecule in the ground state is known to occur and to lead to the depolarization of the fluorescence observed in concentrated solutions (Gaviola & Pringsheim, 1924; Pheofilov & Sveshnikoff, 1941). From such depolarization measurements the distance at which probability of transfer equals probability of emission for parallel oscillators has been found for several dyes to be about 30 Å (Weber, 1954). From the number of tyrosine residues and the dimensions of ordinary globular protein molecules it appears that internal transfer of the excitation energy of the tyrosine must occur. Still more probable appears the transfer from phenylalanine to tryptophan or to tyrosine, from tyrosine to tryptophan and from all the aromatic amino acids to the haem group of haem–proteins. This is no doubt the reason for the high quantum yield of the photodissociation of carboxymyoglobin when illuminated by light absorbed by the aromatic residues of the protein (Bücher & Kaspers, 1946). Such possibilities of transfer place definite limitations on the interpretation of the action spectra of photochemical effects in the complex particulate systems of the living cell.

SUMMARY

1. The fluorescence-excitation spectrum, fluorescence spectrum and quantum yield are proposed as criteria to characterize a molecular species in solution as a fluorescent entity.

2. The fluorescence spectra of phenylalanine, tyrosine and tryptophan in neutral water solution are shown to consist of single bands in the ultraviolet with maxima at 282, 303 and 348 mμ. respectively.

3. The fluorescence-excitation spectra determined from 200 to 320 mμ. correspond accurately to the known absorption spectra of the amino acids, showing the constancy of the quantum yield over the range of wavelengths investigated.

4. The quantum yields (± 1%) in neutral water solutions are 4% for phenylalanine, 21% for tyrosine and 20% for tryptophan.

5. The possibility of electronic energy transfer among the aromatic residues in proteins and to the haem in haemoproteins is discussed.

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The Metabolism of 2:4-, 2:5- and 3:4-Dichloronitrobenzene in the Rabbit

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Previous studies of the metabolism of members of the chloronitrobenzene series (Bray, Hybs, James & Thorpe, 1953; Betts, James & Thorpe, 1955; Bray, James & Thorpe, 1955*b*, 1956) revealed that an appreciable proportion of a dose of most of these compounds was converted into mercapturic acid in the rabbit. These mercapturic acids had been formed by a process resulting in the substitution of either a nitro or a chloro group by the acetylcysteyl group (acetylcysteyldenitration or acetylcysteyldechlorination) instead of replacement of a nuclear hydrogen as had been observed in all previous instances of the formation of nuclear mercapturic acids (for references see Bray *et al.* 1956). Since penta- and two tetra-chloronitrobenzenes yielded mercapturic acids by acetylcysteyldenitration and the monochloronitrobenzenes by acetylcysteyldechlorination it was of interest to examine the intermediate members of the series and to attempt to trace the factors which determined the type of mercapturic acid formed.

The present paper describes experiments on the effect of the administration of three dichloronitrobenzenes on the excretion by the rabbit of mercapturic acid, glucuronic acid, ethereal sulphate and dichloroanilines. In each case the mercapturic acids have been isolated and several phenolic metabolites have been identified. A tetrachloroazoxybenzene has been found in the urine of rabbits given 3:4-dichloronitrobenzene. A preliminary account of some aspects of this work has been given (Bray, James & Thorpe, 1955*a*).

MATERIALS

All melting points recorded are uncorrected.

2:4-, 2:5- and 3:4-Dichloronitrobenzene, m.p. 30°, 56° and 42° respectively, were purchased. 2-Bromo-4-chloro-, 2-bromo-5-chloro- and 4-bromo-3-chloro-nitrobenzene, m.p. 49°, 70° and 61° respectively, were prepared by the action of potassium bromide and copper powder on diazotized 5-chloro-2-nitro-, 4-chloro-2-nitro- and 2-chloro-4-nitro-aniline respectively (cf. Gattermann, 1890). 4-Chloro-2-nitro- and 2-chloro-4-nitro-aniline were purchased and 5-chloro-2-nitroaniline was prepared as described by Hodgson & Kershaw (1929). 2:4-Dichloroacetanilide, m.p. 142°, and 2:5-dichloroacetanilide, m.p. 132–134°, were obtained by acetylation of the corresponding bases.

The following dichloronitrophenols were prepared: 2:4-dichloro-5-nitrophenol, m.p. 105°, as described by Groves, Turner & Sharp (1929); 3:5-dichloro-2-nitrophenol, m.p. 48°, by the method of Hodgson & Wignall (1927) from 3:5-dichloroaniline prepared according to Holleman (1904); 3:6-dichloro-2-nitrophenol, m.p. 67–68°, by the method of Hodgson & Kershaw (1929) from 2:5-dichlorophenol prepared as described by Noelting & Kopp (1905); 2:5-dichloro-4-nitrophenol, m.p. 115°, from 2:5-dichloro-4-nitroaniline (Holleman & Haeften, 1921) by decomposition of the diazo compound according to Noelting & Kopp (1905).

The dichloronitrophenols were reduced to the corresponding aminophenols with alkaline dithionite. The properties of 5-amino-2:4-dichlorophenol and 2-amino-3:5-dichlorophenol were as described by Hodgson & Wignall (1927) and Jacobs, Heidelberger & Rolf (1919) respectively. 2-*Amino*-3:6-*dichlorophenol* formed buff plates, m.p. 137°. (Found N, 7·95. $C_6H_5ONCl_2$ requires N, 7·9%.) 4-Amino-2:5-dichlorophenol, m.p. 174°, was characterized as 4-*acetamido*-2:5-*dichlorophenol*, colourless rosettes of needles from aqueous ethanol, m.p. 208–210°. (Found: N, 6·4. $C_8H_7O_2NCl_2$ requires N, 6·4%.) R_F values in solvents *B* and *C* of Table 2 were 0·59 and 0·64 respectively. The spots were detected by spraying with the Folin & Ciocalteu reagent.

3:4:3′:4′-*Tetrachloroazobenzene* was prepared by keeping 3:4-dichloronitrosobenzene (cf. Vanino, 1923) with an equivalent amount of 3:4-dichloroaniline in acetic acid overnight. It formed orange plates, m.p. 150°. (Found: C, 44·8; H, 1·6; N, 8·7; Cl, 44·6. $C_{12}H_6N_2Cl_4$ requires C, 45·0; H, 1·9; N, 8·8; Cl, 44·3%.) 3:4:3′:4′-*Tetrachloroazoxybenzene*, m.p. 139°, was prepared (*a*) by oxidation of 3:4:3′:4′-tetrachloroazobenzene in ethanol–acetic acid (1:1, v/v) by the daily addition of 2 g. of urea hydrogen peroxide (British Drug Houses Ltd.) for 7 days, or (*b*) by boiling 3:4-dichlorophenylhydroxylamine in 96% (w/v) ethanol for 15 min. (For analysis see below.) 3:4-Dichlorophenylhydroxylamine was prepared from 3:4-dichloronitrobenzene as described by Haworth & Lapworth (1921).

N-*Acetyl*-S-(5-*chloro*-2-*nitrophenyl*)-L-*cysteine*, m.p. 192°, N-*acetyl*-S-(4-*chloro*-2-*nitrophenyl*)-L-*cysteine*, m.p. 161°, and N-*acetyl*-S-(2-*chloro*-4-*nitrophenyl*)-L-*cysteine*, m.p. 193°, were prepared by the method of Parke & Williams (1951) from 5-chloro-2-nitroaniline, 4-chloro-2-nitroaniline and 2-chloro-4-nitroaniline respectively. The bases were diazotized in conc. H_2SO_4 as described for the preparation of *N*-acetyl-*S*-(2-nitrophenyl)-L-cysteine (Bray *et al.* 1956). Properties and elementary analyses are given in Table 1. The three mercapturic acids were also prepared by the method of Suter (1895) with ethylene glycol monoethyl ether as solvent. This is a more convenient method but it does not provide proof of the constitution of the acid formed.

Table 1. *Properties of three chloronitrophenylmercapturic acids*

	N-Acetyl-*S*-(5-chloro-2-nitrophenyl)-L-cysteine	*N*-Acetyl-*S*-(4-chloro-2-nitrophenyl)-L-cysteine	*N*-Acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine	$C_{11}H_{11}O_5N_2ClS$ requires
Cryst. form	Rosettes of yellow needles	Yellow needles	Cream needles	
Melting point	192°	161–162°	192–194°	—
$[\alpha]_D^{20}$ in ethanol (*c*, 0·5)	+63±3°	+104±7°	−4±4°	—
Found:				
C (%)	41·5*	41·3	41·6	41·5
H (%)	3·6	3·3	3·5	3·5
N (%)	8·3	8·8	9·2	8·8
Cl (%)	11·3	11·2	10·7	11·1
S (%)	9·8	9·9	9·9	10·1
Equiv.	310	314	314	319
Absorption spectra in 0·1 N-KOH				
$\lambda_{max.}$ (mμ.)	253, 375	249, 390	345	—
$\epsilon_{max.}$ (synthetic)	13 540, 3156	17 380, 2815	10 025	—
$\epsilon_{max.}$ (biosynthetic)	14 420, 3406	16 903, 2806	10 010	—
$\lambda_{min.}$ (mμ.)	230, 328	226, 315	276	—
Inflexion (mμ.)	285	270	—	—

* Mean of triplicate analysis.

METHODS

Animals, diet and dosage. Doe rabbits (2–3 kg. body wt.) were maintained as previously described (Bray, Ryman & Thorpe, 1947). All compounds were administered by stomach tube as suspensions in water. The dose levels were: 2:4-isomer, 0·2, 0·3 and 0·4 g./kg.; 2:5- and 3:4-isomer, 0·4 g./kg. There was pronounced anorexia after giving 0·4 g. of the 2:4-isomer/kg.

Determination of metabolites

Ethereal sulphate. The method of Folin (1905–6) was used.

Glucuronic acid. A modification (Bray, Humphris, Thorpe, White & Wood, 1952) of the naphthoresorcinol method of Hanson, Mills & Williams (1944) was used. Reducing material was determined as described by Bray, Neale & Thorpe (1946).

Ether-soluble acid. The method of Bray *et al.* (1946) was used. The recovery of *N*-acetyl-*S*-(5-chloro-2-nitrophenyl)-L-cysteine added to urine was 109±16%.

Mercapturic acid. All the mercapturic acids formed were determined by the modified Stekol (1936) method described by Betts *et al.* (1955). The concentration of NaOH used for hydrolysis and time of boiling were 0·5 N and 0·5 hr. for *N*-acetyl-*S*-(5-chloro-2-nitrophenyl)-L-cysteine and 2 N and 1 hr. for *N*-acetyl-*S*-(4-chloro-2-nitrophenyl)-L-cysteine and *N*-acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine. The recoveries from urine were 46, 49 and 31% (all±5) respectively. *N*-Acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine was also determined by the colorimetric method described for *N*-acetyl-*S*-(*p*-nitrophenyl)-L-cysteine (Bray *et al.* 1956). This gave 88±6% recovery of the acid added to urine.

Dichloroanilines. These were determined, on the steam-distillates of urine samples adjusted to pH 10 or of whole 72 hr. faeces, by the method of Bratton & Marshall (1939) with the appropriate bases as standards and a Spekker photoelectric absorptiometer with a Chance glass filter OG. 1. Recoveries of 2:4-, 2:5- and 3:4-dichloroaniline added to urine were 88, 100 and 100% respectively.

Catechols. The method of Azouz, Parke & Williams (1953) was used with 4-chlorocatechol as standard.

Qualitative examination of urines

Ether extracts A, B, C, D and E were prepared from urines as described by Bray *et al.* (1956). Briefly, the extracts were: A, from urine at pH 7–8; B, from the residual urine from A adjusted to pH 1; C, from the residual urine from B hydrolysed with an equal volume of 10 N-H_2SO_4; D, from residual urine from C adjusted to pH 7; E, from hydrolysed urine adjusted to pH 7, was prepared only from urines of rabbits given dichloroanilines.

Paper chromatography. The procedure was as described by Bray, Thorpe & White (1950) except that papers were dried without heating to minimize decomposition of aminophenols. The solvents and detecting reagents used and the R_F values of reference compounds are given in Table 2.

Isolation of metabolites

Unless stated otherwise all metabolites identified were shown to be identical with synthetic specimens by mixed m.p. and paper chromatography.

Dichloroanilines. The urine was adjusted to pH 10 and steam-distilled. The distillate was continuously extracted with ether. The residue left after removal of ether from the extract either yielded the crystalline dichloroaniline or was treated with acetic anhydride to give the dichloroacetanilide which was recrystallized from aqueous ethanol.

Mercapturic acids. The urine was adjusted to pH 1 and continuously extracted with ether. Crystals of the mercapturic acid were isolated from the extract and recrystallized from aqueous ethanol. Results for elementary analyses, optical rotation and absorption spectra are given in Table 1.

Preparation of 3:3′-dichloro-4:4′-dinitrodiphenyl disulphide. *N*-Acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine (0·5 g.) was boiled for 1 hr. in N_2 with 2 N-NaOH (40 ml.). The solution was cooled and acidified, when crystals m.p. 68–71° separated. These were immediately oxidized with ethanolic iodine to give yellow needles of 3:3′-*dichloro*-4:4′-

Table 2. *R_F values and detection of some possible metabolites of* 2:4-, 2:5- *and* 3:4-*dichloronitrobenzene*

Solvent mixtures and times of run on Whatman no. 4 paper: *A*, benzene–acetic acid–water (2:2:1, by vol.) (1·5 hr.); *B*, light petroleum (b.p. 90°)–benzene–98% formic acid (3:1:2, by vol.) (1 hr.); *C*, *n*-butanol–3N-$(NH_4)_2CO_3$–3N-NH_3 soln. (4:3:3, by vol.; Corner & Young, 1954) (2 hr.). Detecting reagents: *a*, 2N-HCl, 0·1% $NaNO_2$, 0·5% ammonium sulphamate and 0·1% *N*-(1-naphthyl)ethylenediamine dihydrochloride (Bratton & Marshall, 1939); *b*, diazotized *p*-nitroaniline followed by 20% (w/v) Na_2CO_3 (Bray *et al.* 1950); *c*, 20% (w/v) Na_2CO_3. Colours of spots: B, blue; G, green; M, magenta; Oc, ochre; P, purple; R, red; Y, yellow; p, pale. The compounds in parentheses were not available as reference compounds and were observed only as metabolites in urine. The arguments for the suggested constitutions are given in the Results section.

Compound	R_F values in solvent mixture			Colour with detecting reagents		
	A	*B*	*C*	*a*	*b*	*c*
Possible metabolites of 2:4-dichloronitrobenzene						
5-Amino-2:4-dichlorophenol	0·76	—	0·70	M	Oc	—
2-Amino-3:5-dichlorophenol	0·89	—	0·83	Y*	P	—
(3-Amino-2:6-dichlorophenol)	0·50	—	—	pR	—	—
2:4-Dichloro-5-nitrophenol	—	0·64	—	—	—	pY
3:5-Dichloro-2-nitrophenol	—	1·0	—	—	—	pY
2:4-Dichloroaniline	1·0	—	0·95	M	Y	—
N-Acetyl-*S*-(5-chloro-2-nitrophenyl)-L-cysteine	0·73	0·0	0·58	—	—	pY
Possible metabolites of 2:5-dichloronitrobenzene						
2-Amino-3:6-dichlorophenol	1·0	—	0·69	Y*	P	—
(3-Amino-2:5-dichlorophenol)	0·95	—	0·83	pR	—	—
4-Amino-2:5-dichlorophenol	0·72	—	0·77	B	pG	—
2:5-Dichloro-4-nitrophenol	—	0·55	—	—	—	pY
3:5-Dichloro-2-nitrophenol	—	1·0	—	—	—	Y
2:5-Dichloroaniline	1·0	—	0·95	M	Y	—
N-Acetyl-*S*-(4-chloro-2-nitrophenyl)-L-cysteine	0·78	0·0	0·59	—	—	pY
Possible metabolites of 3:4-dichloronitrobenzene						
3:4-Dichloroaniline	1·0	—	0·95	M	Y	—
N-Acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine	0·55	0·0	0·77	—	—	Y
(2-Aminodichlorophenol X)†	0·66	—	0·86	B*	—	—
(2-Aminodichlorophenol Y)†	0·72	—	0·75	Y*	—	—
(5-Amino-2:3-dichlorophenol)	0·75	—	0·86	pR	—	—

* Gave bright yellow colour with nitrous acid. † See text under 'Isolation of metabolites'.

dinitrodiphenyl disulphide, m.p. 163–164° after recrystallization from acetic acid. A further yield was obtained from the mother liquor. Total yield, 180 mg. (Found: C, 38·1; H, 1·3; N, 7·5; Cl, 18·6; S, 16·5. $C_{12}H_6O_4N_2Cl_2S_2$ requires C, 38·2; H, 1·6; N, 7·4; Cl, 18·8; S, 17·0%.)

3:4:3′:4′-*Tetrachloroazoxybenzene*. The ether extract from urine (pH 1) of rabbits dosed with 3:4-dichloronitrobenzene contained tetrachloroazoxybenzene in addition to the mercapturic acid. Ether was removed from the extract and the residue was extracted with dilute NaOH to remove the mercapturic acid. The insoluble material left was washed with water and recrystallized from ethanol to give pale-yellow needles of 3:4:3′:4′-*tetrachloroazoxybenzene*, m.p. 139° (not examined by paper chromatography). (Found: C, 42·8; H, 1·8; N, 8·5; Cl, 42·2. $C_{12}H_6ON_2Cl_4$ requires C, 42·9; H, 1·8; N, 8·3; Cl, 42·2%.)

4-*Amino*-2:5-*dichlorophenol*. The urine was refluxed for 1 hr. with an equal volume of 10N-H_2SO_4 and was then made alkaline and steam-distilled to remove the dichloroaniline. The residual urine was adjusted to pH 6 and continuously extracted with ether. On evaporation of the ether, crude aminophenol was obtained, m.p. 160–170° after recrystallization from ethanol–light petroleum (b.p. 40–60°). This gave a positive indophenol test for a *p*-aminophenol and a blue colour with the Bratton & Marshall reagents. Acetylation with acetic anhydride at room temperature gave 2:5-dichloro-4-hydroxyacetanilide, m.p. 208°.

2-*Aminodichlorophenol X*. The urine was continuously extracted with ether at pH 1. The mercapturic acid was removed from the extract by extraction with N-$NaHCO_3$ and the remaining ethereal solution was extracted with 0·5N-NaOH. The extract was adjusted to pH 6 and re-extracted with ether. Evaporation of the ether gave a syrup, which rapidly darkened if kept. It formed with acetic anhydride a crystalline acetyl derivative, m.p. 220° (softening at 214°) after recrystallization from aqueous ethanol. Yield, 30 mg. (Found: C, 44·0; H, 3·5; N, 6·3. $C_8H_7O_2NCl_2$ requires C, 43·7; H, 3·2; N, 6·4%.) This aminophenol (prepared by hydrolysis of the acetyl derivative) when treated with the Bratton & Marshall (1939) reagents gave a bright yellow colour at the nitrous acid stage. When the coupling reagent was added the colour quickly changed to blue, and the blue dye was soon deposited from the solution.

RESULTS

The average daily excretions of normal metabolites by the rabbits used were of the same order as those found previously (e.g. Bray *et al.* 1953). The results

Table 3. *Excretion of metabolites of* 2:4-, 2:5- *and* 3:4-*dichloronitrobenzene by the rabbit*

Results are expressed as percentages of the dose, given as means with ranges in parentheses; superior figures indicate the number of experiments. A superior number followed by p indicates a result for pooled urine from that number of rabbits. Urine was collected for 72 hr. except in experiments (indicated by m after the superior number) where separate consecutive samples, which were collected from rabbits given water at intervals (see Bray, Thorpe & White, 1951), were analysed until the excretion of metabolites was no longer detected. From 67 to 80% of the unabsorbed material in faeces was reduced to the dichloroaniline. The reducing value of the urine after feeding the 2:4-isomer increased to an extent corresponding to 62% of the glucosiduronic acid excreted and for the 2:5- and 3:4-isomers the increase was equivalent to the glucosiduronic acid excreted (3 expts. with each isomer).

	2:4-Dichloronitrobenzene			2:5-Dichloronitrobenzene	3:4-Dichloronitrobenzene
Dose (g.)	0·5	0·7	1·0	1·0	1·0
Metabolite					
Unabsorbed	—	—	0·3 (0·3, 0·3)^{2}	1·1 (0·5, 1·7)^{2}	0·2 (0·1, 0·4)^{2}
Mercapturic acid	84 (61–98)^{6}	67 (24–91)^{6} 49 (20–72)^{6*}	53 (38–67)^{5} —	27 (9–33)^{7} —	39 (16–70)^{9} 45 (30–56)^{6†}
Glucosiduronic acid	22 (17, 26)^{2m}	—	5 (0–13)^{10}	29 (8–56)^{14}	13 (7–23)^{7}
Ethereal sulphate	5 (0–14)^{3m}	—	6 (0–12)^{8}	14 (3–21)^{5}	12 (9–13)^{3}
Dichloroaniline, free	6 (6, 6)^{2m}	—	8^{3p}	15 (10–19)^{12}	17 (8–23)^{12}
Dichloroaniline, combined	—	—	3^{3p}	1^{10p}	5 (0–11)^{9}
'Catechols'	—	—	5 (4, 6)^{2}	6 (1–9)^{3}	2 (1–2)^{3}
Azoxy compound	—	—	—	—	2^{6p‡}
Total accounted for, (excluding 'catechols')	—	—	78	87	94

* Determined as ether-soluble acid. (Not the same animals as for Stekol method.)
† Colorimetric method. ‡ By isolation.

obtained from the quantitative analysis of urines are summarized in Table 3, which shows that with each compound a satisfactory proportion of the dose has been accounted for. In each case the greater part of the dose was excreted as mercapturic acid and phenolic metabolites. Relatively little was excreted as the dichloroaniline. The totals do not include the small amounts of phenolic metabolites found unconjugated in urine (Table 4, extracts A and B). These may have been formed by hydrolysis of glucosiduronic acids, which are presumably labile since there was good agreement between the results of glucuronic acid and reducing-value determinations. The values for mercapturic acids by the Stekol method have been corrected according to recoveries obtained when the pure acids were added to urine. With the 3:4-isomer the validity of the correction is supported by the results of analyses by the colorimetric method. The colour given by the other mercapturic acids with NaOH was too feeble for satisfactory quantitative determination by this method. The presence of the small amounts of catechol derivatives indicated by quantitative determination has not been confirmed by either isolation or detection of catechol derivatives. These amounts have not, therefore, been included in the totals accounted for. Unchanged dichloronitrobenzenes were apparently absent from the urines, since the amounts of chloroanilines found were not increased after treating the urine with zinc and hydrochloric acid.

Metabolites identified

The metabolites identified by paper chromatography are listed in Table 4. No nitro- or aminomonochlorophenols and no acetamido compounds other than the mercapturic acids were detected. No nitrophenols were detected after administration of 3:4-dichloronitrobenzene. Table 4 includes the metabolites detected by paper chromatography in urines from rabbits dosed with 2:4-, 2:5- and 3:4-dichloroaniline.

Metabolites isolated

The yields of the metabolites isolated are given in Table 5. A tetrachloroazoxybenzene was found only in the urines of animals dosed with 3:4-dichloronitrobenzene. The constitution of the 2-aminodichlorophenol X isolated from such urines was not fully established. The intense yellow colour given on treatment with nitrous acid points to its being an *o*-aminophenol, i.e. either 2-amino-4:5-dichlorophenol or 6-amino-2:3-dichlorophenol. (When 3:4-dichloroaniline was given, the other *o*-aminophenol, 2-aminodichlorophenol Y, was excreted.) 5-Amino-2:3-dichlorophenol is suggested as the probable constitution of the other phenolic metabolite (R_F 0·75 in solvent *A*) since this gave no yellow colour with nitrous acid and it is the only other possible aminodichlorophenol from 3:4-dichloronitrobenzene. The aminomonochlorophenol which would be expected if a chlorine atom were

Table 4. *Metabolites of dichloronitrobenzenes and dichloroanilines detected by paper chromatography*

Metabolites of dichloronitrobenzenes were sought in extracts A, B, C and D, and of dichloroanilines in extract E. For preparation of extracts see Methods. Compounds found in extracts of hydrolysed urine (C and D) are probably excreted in combined form. +, Present; (+), present in traces only; −, absent.

	Detected in extract				
	A	B	C	D	E
Metabolites of 2:4-isomer					
2:4-Dichloroaniline	+	+	+	+	+
2:4-Dichloro-5-nitrophenol	+	+	(+)	(+)	−
3:5-Dichloro-2-nitrophenol	+	+	(+)	−	−
2-Amino-3:5-dichlorophenol	(+)	(+)	(+)	+	+
3-Amino-2:6-dichlorophenol*	(+)	−	−	−	−
5-Amino-2:4-dichlorophenol	(+)	(+)	(+)	(+)	−
N-Acetyl-*S*-(5-chloro-2-nitrophenyl)-L-cysteine	−	+	−	−	−
Metabolites of 2:5-isomer					
2:5-Dichloroaniline	+	+	(+)	−	+
2:5-Dichloro-4-nitrophenol	−	(+)	−	−	−
3-Amino-2:5-dichlorophenol*	−	(+)	−	−	−
4-Amino-2:5-dichlorophenol	(+)	(+)	−	+	+
N-Acetyl-*S*-(4-chloro-2-nitrophenyl)-L-cysteine	−	+	−	−	−
Metabolites of 3:4-isomer					
3:4-Dichloroaniline	+	+	+	+	+
2-Aminodichlorophenol X*†	(+)	(+)	(+)	+	+
2-Aminodichlorophenol Y*†	−	−	−	−	−
5-Amino-2:3-dichlorophenol*	−	+	−	+	−
N-Acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine	−	+	−	−	−

* Reference compounds not available. See Results section for arguments for the suggested constitutions.
† Probably either 2-amino-4:5- or 2-amino-5:6-dichlorophenol (see text).

Table 5. *Metabolites isolated from urine of rabbits given dichloronitrobenzenes and bromochloronitrobenzenes*

Nitrobenzene derivative administered	Dose/rabbit (g.)	No. of rabbits	Compound isolated	Yield (% of dose)
2:4-Dichloro-	0·7	6	2:4-Dichloroaniline (as acetyl deriv.)	1
		6	*N*-Acetyl-*S*-(5-chloro-2-nitrophenyl)-L-cysteine	23
2:5-Dichloro-	1	10	2:5-Dichloroaniline	13
		10	*N*-Acetyl-*S*-(4-chloro-2-nitrophenyl)-L-cysteine	2
		6	4-Amino-2:5-dichlorophenol	1
3:4-Dichloro-	1	10	3:4-Dichloroaniline	5
		12	*N*-Acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine	17
		6	3:4:3′:4′-Tetrachloroazoxybenzene	2
		12	2-Aminodichlorophenol X (as acetyl deriv.)	1
2-Bromo-4-chloro-	0·5	2	*N*-Acetyl-*S*-(5-chloro-2-nitrophenyl)-L-cysteine	10
2-Bromo-5-chloro-	0·5	2	*N*-Acetyl-*S*-(4-chloro-2-nitrophenyl)-L-cysteine	1
4-Bromo-3-chloro-	0·5	2	*N*-Acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine	7

replaced by a hydroxyl group would be 4-amino-3-chlorophenol, since the chlorine on C-4 of 3:4-dichloronitrobenzene is the more labile one (Holleman, Mooy & Weel, 1915). This aminochlorophenol has in solvent *A* an R_F value of 0·23 (Bray *et al.* 1956) which is much lower than that of the metabolite detected (R_F 0·75). Similar arguments apply for the attribution of the probable constitutions of 3-amino-2:6-dichlorophenol and 3-amino-2:5-dichlorophenol to the aminophenols detected in urine after administration of 2:4- and 2:5-dichloronitrobenzene respectively. Table 5 includes the mercapturic acids isolated from urines of rabbits dosed with bromochloronitrobenzenes, which were given in order to provide additional evidence for the constitutions of the mercapturic acids formed from the dichloronitrobenzenes.

DISCUSSION

The most conspicuous quantitative difference between the metabolic fates of the three dichloronitrobenzenes and those of the monochloronitrobenzenes (Bray *et al.* 1956) is that in the former mercapturic acid formation provides the major metabolic pathway, whereas with the latter only

small amounts of mercapturic acid are excreted and hydroxylation is the principal metabolic process. The excretion of chloroanilines in the two groups is quantitatively similar. In both cases a relatively small proportion was excreted in combined form but acetylation could not be detected.

It is difficult to predict the position of hydroxylation in the three dichloronitrobenzenes, since in the same nucleus there are two *ortho–para*-directing, but deactivating, chloro groups together with a nitro group. The aminodichlorophenols detected in urines of rabbits dosed with the dichloroanilines are those which would be expected from a dominating *ortho–para*-directing amino group. The hydroxylation products detected from the dichloronitrobenzenes and dichloroanilines have been summarized in Fig. 1, which shows, on the basis of the existing evidence, the probable stage at which hydroxylation occurs. It seems likely, for example, that 5-amino-2:4-dichlorophenol is a product of the reduction of 2:4-dichloro-5-nitrophenol rather than a product of the hydroxylation of 2:4-dichloroaniline, since the aminophenol is a *m*-aminophenol which was not detected as a metabolite of the dichloroaniline. 2-Amino-3:5-dichlorophenol, on the other hand, could have been formed either by reduction of 3:5-dichloro-2-nitrophenol or by hydroxylation of 2:4-dichloroaniline.

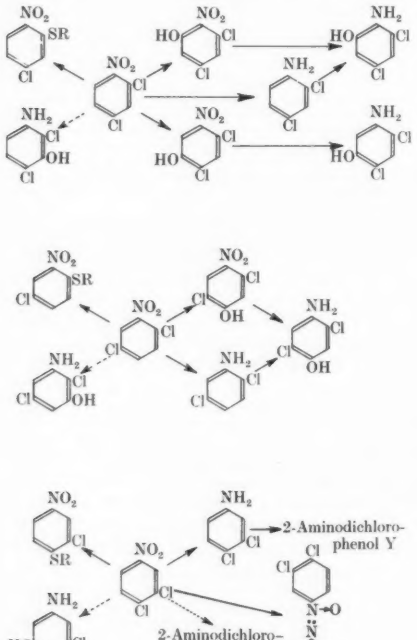

Fig. 1. Metabolites excreted in urine by rabbits dosed with 2:4-, 2:5- and 3:4-dichloronitrobenzene and 2:4-, 2:5- and 3:4-dichloroaniline. The broken arrows point to aminophenols, which were probably formed from nitrophenols not detected in the urines and which were not found as metabolites of the dichloroaniline. Aminodichlorophenol Y was not detected as a metabolite of 3:4-dichloronitrobenzene.

The most interesting feature of the metabolism of the three dichloronitrobenzenes is the nature of the mercapturic acids formed. All the mercapturic acids have retained the nitro group and one of the chloro groups has been substituted by an acetylcysteyl group. The process is, in effect, one of acetylcysteyldechlorination. In certain higher members of the chloromononitrobenzene series (penta- and some tetra-chloronitrobenzenes) it has been shown that mercapturic acid formation was, in effect, an acetylcysteyldenitration (Bray *et al.* 1953; Betts *et al.* 1955). A preliminary examination of some trichloronitrobenzenes has shown that these compounds appear to form mercapturic acid by acetylcysteyldechlorination (Bray *et al.* 1955*b*). The reason for the formation of mercapturic acids by substitution of chloro or nitro groups rather than by the more usual process of acetylcysteyldeprotonation observed in aromatic hydrocarbons and the halogenobenzenes (for references see Bray *et al.* 1956) seems to be related to the lability of the groups displaced by the acetylcysteyl residue. The correspondence between the ease of hydrolysis of the nitro groups by alkali in some polychloronitrobenzenes and the extent of mercapturic acid formation in the rabbit has already been reported (Betts *et al.* 1955). A similar relation for the displaced chloro group of the dichloronitrobenzenes is shown in Table 6, in which the rate coefficients for hydrolysis with sodium methoxide (determined by Holleman *et al.* 1915) are compared with the amounts of mercapturic acids excreted by the rabbit. Table 6 also shows that the mercapturic acids formed have the acetylcysteyl group in the position occupied by the labile chlorine in the original chloronitrobenzene. The structures of all three mercapturic acids have been established by comparison with the acids synthesized via the diazonium salts of the corresponding chloronitroanilines. This method of synthesis leaves no doubt as to their structure. Further evidence that the mercapturic acids are formed by substitution of the labile chlorine atom is provided by the experiments with bromochloronitrobenzenes. In these compounds bromine is more labile than chlorine and the bromine occupies the position of the labile chlorine in

Table 6. *Mercapturic acid formation and chloride mobility in some chloromononitrobenzenes*

The positions of the labile chlorine atoms and their mobilities (rate coefficients for hydrolysis with methanolic sodium methoxide at 25°) are those given by Holleman *et al.* (1915). In column 3, *m* represents mobility of labile chlorine.

Nitrobenzene derivative	Position of labile halogen	10^5m (l. mole^{-1} min.$^{-1}$)	Mercapturic acid formed by rabbit: Amount (% of dose) By quantitative analysis	Amount (% of dose) By isolation	Position of acetylcysteyl group relative to nitro group
2:4-Dichloro-	2	50	53	23	2
2-Bromo-4-chloro-	2	—	—	10	2
2:5-Dichloro-	2	10	27	2	2
2-Bromo-5-chloro-	2	—	—	1	2
3:4-Dichloro-	4	55	45	17	4
4-Bromo-3-chloro-	4	—	—	7	4
o-Chloro-	2	1*	7	—	2
m-Chloro-	Stable	0	0	—	—
p-Chloro-	4	4*	2	—	4

* Interpolated from values at 85° and 110°.

the corresponding dichloro compound. In each case the mercapturic acid isolated from urine after giving a bromochloronitrobenzene to rabbits was the same as that obtained after giving the corresponding dichloronitrobenzene. Although a considerable part of each dichloronitrobenzene was reduced in the rabbit to amino compounds, no *N*-acetyl-*S*-(aminochlorophenyl)-L-cysteine was ever detected.

While theoretically the reduction of a nitro group would be expected to involve the intermediate formation of nitroso and hydroxylamino compounds, the biological production of such compounds has been difficult to demonstrate. If these intermediates were produced *in vivo*, it would be expected that the corresponding azoxy or azo compounds might be formed. Channon, Mills & Williams (1944), however, showed that the tetranitroazoxytoluene, which was found in the urine of subjects with 2:4:6-trinitrotoluene poisoning, was an artifact formed from 4-hydroxylamino-2:6-dinitrotoluene when the urine was kept. The origin of the tetrachloroazoxybenzene found in the urine of rabbits after administration of 3:4-dinitrochlorobenzene is, therefore, of interest. So far it has not been possible to prove that it is an artifact and attempts to detect or measure the excretion of hydroxylamino compounds in freshly voided urine have not been successful, partly because 3:4-dichloroaniline does not react readily with acetic anhydride under the conditions of the Rosenthal & Bauer (1939) method of determination. The Pucher & Day (1926) reaction for hydroxylamine was also negative with 3:4-dichlorophenylhydroxylamine. The formation of the 3:4-3′4′-tetrachloroazoxybenzene could not be detected when 3:4-dinitrophenylhydroxylamine was added to urine under the conditions used by Channon *et al.* (1944), although the azoxybenzene was easily isolated from the urine of rabbits dosed with 3:4-dichloronitrobenzene. While it is probable that the azoxy compound is derived from an intermediate in the reduction of the nitrocompound there is as yet no evidence to show how or where it is formed. The available evidence suggests that the azoxy compound is not an artifact.

SUMMARY

1. The metabolism of 2:4-, 2:5- and 3:4-dichloronitrobenzene has been studied in the rabbit.

2. The main products excreted in urine are mercapturic acids (corresponding to 30–50% of the dose) and phenols conjugated with glucuronic and sulphuric acids. Smaller amounts of dichloroanilines are excreted.

3. The mercapturic acids have been isolated and their structure has been confirmed by synthesis. In each case they are formed by substitution of the labile chlorine atom.

4. 2-Bromo-4-chloro-, 2-bromo-5-chloro- and 4-bromo-3-chloro-nitrobenzene when fed to rabbits yield the same mercapturic acids as do the corresponding dichloronitrobenzenes.

5. Several of the nitro- and amino-dichlorophenols excreted have been identified.

6. 3:4:3′:4′-Tetrachloroazoxybenzene has been isolated from the urine of rabbits given 3:4-dichloronitrobenzene.

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Boundary Spreading in Sedimentation-Velocity Experiments

4. MEASUREMENT OF THE STANDARD DEVIATION OF A SEDIMENTATION-COEFFICIENT DISTRIBUTION: APPLICATION TO BOVINE ALBUMIN AND β-LACTOGLOBULIN*

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Methods of studying the heterogeneity of proteins have been essential to the modern development of protein chemistry. One of the most useful methods has been analysis by means of the velocity ultracentrifuge, in which molecular species of different size, shape or density are resolved. This article provides a method for measuring the standard deviation of a sample's distribution of sedimentation coefficient. One would prefer to measure the distribution itself, but for several systems this is not possible while measurement of the standard deviation, p, of the distribution is possible.

One reason for studying the heterogeneity of a protein preparation is to find out whether homogeneous proteins exist in Nature. Because of the possibility of changes produced during fractionation or upon storage, measurements of heterogeneity must be interpreted with great caution. This subject has been reviewed by Pirie (1940) and recently by Colvin, Smith & Cook (1954). Another reason for studying the heterogeneity of a protein sample is to aid in interpreting other physical measurements. For example, heterogeneity enters into the measurements of sedimentation coefficient, diffusion coefficient and intrinsic viscosity in different ways, so that, in interpreting these measurements by a theory such as that of Scheraga & Mandelkern (1953) for the hydrodynamic behaviour, one must have detailed knowledge of the heterogeneity. A brief review of the measurement of heterogeneity by diffusion, electrophoresis

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and sedimentation experiments is given by Baldwin, Gosting, Williams & Alberty (1955*b*).

The general purpose of this series of papers has been to develop methods for the measurement of heterogeneity in the sedimentation behaviour of proteins and of other biological macromolecules. The first paper (Williams, Baldwin, Saunders & Squire, 1952) presented in outline a theory for taking into account the effects of diffusion on the measurement of heterogeneity when the distribution of sedimentation coefficient is continuous. The ordinate of the differential distribution curve was denoted by g(S), the sedimentation coefficient being S. (In the preceding papers of this series, sedimentation coefficient has been denoted by s.) It was pointed out that, if concentration dependence effects could be ignored, then the curve of g(S) against S could be obtained by extrapolation of an 'apparent distribution' to infinite time and also an independent determination could be made of the distribution's standard deviation, p, from the variation with time of σ^2, the second moment about the mean of the boundary-gradient curve. Concurrently, Gosting (1952) gave a full theoretical account of the extrapolation to infinite time which is used in finding g(S). Studies of dextran (Williams & Saunders, 1954) and gelatin (Williams, Saunders & Cicirelli, 1954) systems showed that, in general, concentration-dependence effects must be taken into account in finding g(S) by means of a further extrapolation to infinite dilution. The second paper (Baldwin, 1954*a*) treated this problem by an approximate theory, and a method was developed for using the results of only one experiment to find g(S), by means of a calculated correction for the effects of concentration dependence. This method was used by Ogston & Woods (1954) in their study of various dextrans.

There is a limit to the resolving power of present ultracentrifuges, and this restricts the class of systems for which g(S) may be found by extrapolation to infinite time. This problem was investigated in the third paper (Baldwin, 1954*b*) by means of trial extrapolations to infinite time based on an analytical expression for the case that g(S) is Gaussian. For a given field strength and duration of the experiment, the accuracy of the extrapolated distribution is related to the ratio of heterogeneity to diffusion coefficient for the system. It was found that, for many systems in which heterogeneity significantly influenced the spread of the boundary-gradient curves, the resolving power was too low for g(S) to be found by extrapolation. Also, when the distribution of S is discontinuous, for example, when there is a discrete mixture of solutes whose boundary-gradient curves overlap, it is not yet possible to obtain the distribution by extrapolation to infinite time.

No such limitation affects the measurement of p from the variation of σ^2 with time; so long as heterogeneity measurably affects the spread of the boundary-gradient curves, one can calculate p. However, the dependence of S on concentration (c) is not included in the equation for finding p given by Williams *et al.* (1952) (see also Baldwin, 1954*b*). Experiments (Baldwin, 1953*a*) have shown that this is a serious limitation. An approximate, unpublished theory (Baldwin, 1953*a*) which takes into account the concentration-dependence effect has been quoted by Field & O'Brien (1955) and has been used (Baldwin, 1953*c*) in studying the neurotoxin of *Shigella shigae* prepared by Van Heyningen & Gladstone (1953). It is this theory, in a more rigorous form, which will be presented here. The end result is an equation for σ^2 which takes into account the dependence of S on c and which can be used to find p. The case considered is that of a heterogeneous system in which both the sedimentation and diffusion coefficients of the individual species are functions only of the total concentration. (Because of the demands of experiment, concentration is expressed on a refractive-increment basis.)

THEORY

Since p can be found from σ^2, the second moment about the mean of $\partial n_c/\partial x$ against x, for the case in which the sedimentation and diffusion coefficients do not vary with concentration (Williams *et al.* 1952; Baldwin, 1954*b*), it was natural to hope that the concentration-dependent case could be solved by finding an expression for σ^2. With this purpose in mind, a fairly general method has been developed for finding moments of the boundary-gradient curves observed in sedimentation, diffusion and electrophoretic experiments. It has been used (Baldwin, 1954*b*), when Faxén's (1929) equation for the sedimentation of a single solute applies, to find a simple expression in closed form for the positive even moments of the gradient curves. (Faxén's equation itself is expressed in an infinite series.) The method has also been used (Baldwin, Dunlop & Gosting, 1955*a*) in studying the diffusion of several solutes whose flows interact.

In outline, the procedure is as follows: First one writes an expression for any moment

$$(\mu_r,\ r=0,\ 1,\ 2,\ \ldots)$$

of the curve of $\partial n_c/\partial x$ against x in terms of the individual concentration gradient curves. This is then differentiated with respect to time. Next, by means of the expression for conservation of mass, one introduces the appropriate flow equation for each solute and thus its sedimentation and diffusion coefficients. The resulting expression is a differential equation for the rate of change of any specified

moment with time. This equation is put into suitable form for integrating with respect to time and the final step is integration.

Definition of p and $\overline{S}_0$, the standard deviation and the mean of the distribution of S_0. The optical systems now used for ultracentrifugal analysis (cf. Philpot, 1938; Svensson, 1939; Philpot & Cook, 1948) record, as a function of position in the cell x, a quantity related linearly to n, the refractive index of the solution, or to its gradient $(\partial n/\partial x)_t$. In this paper it will be assumed that n can be adequately represented by the equation

$$n = n_0 + \sum_i R_i c_i, \tag{1}$$

where n_0 is the refractive index of the solvent at the same temperature and pressure (the solvent will be taken to include dialysable constituents such as buffer and salt ions), c_i is the volume concentration of the macromolecular solute i (in g./100 ml. of solution) and R_i is the differential refractive increment of i:

$$R_i \equiv \left(\frac{\partial n}{\partial c_i}\right)_{T,\,P,\,c_{j\neq i}}, \tag{2}$$

where T is the temperature, P is the pressure and j includes all macromolecular species other than i. In the sedimentation experiment, the temperature is assumed to be constant and R_i will be taken to be constant, independent of pressure and of the concentrations of the various solute species. Thus the gradient of n (i.e. its partial derivative with respect to x at constant t) is written

$$\left(\frac{\partial n}{\partial x}\right)_t = \left(\frac{\partial n_0}{\partial x}\right)_t + \sum_i R_i \left(\frac{\partial c_i}{\partial x}\right)_t, \tag{3}$$

where $(\partial n_0/\partial x)_t$ includes the gradient caused by buffer sedimentation. Hereafter the subscript t will be omitted. In experimental practice, reference base lines are used to subtract (approximately) $\partial n_0/\partial x$ from $\partial n/\partial x$; in any case it will be assumed here that $\sum_i R_i c_i$ (or its gradient) is readily measurable. For brevity, this quantity will be denoted by n_c:

$$n_c \equiv \sum_i R_i c_i, \tag{4a}$$

$$\frac{\partial n_c}{\partial x} = \sum_i R_i \frac{\partial c_i}{\partial x}. \tag{4b}$$

Since refractive increments are measured experimentally, it is convenient to define the heterogeneity of the sample on a refractive-increment basis. For the case that the sample contains a set of discrete components, the fractional amount of solute i, α_i, may be written

$$\alpha_i \equiv R_i c_i^0 / \sum_i R_i c_i^0, \tag{5}$$

where c_i^0 is the initial concentration of i before any sedimentation has taken place. Then, on this refractive increment basis, p is defined by

$$p^2 \equiv \sum_i \alpha_i (S_{i,\,0} - \overline{S}_0)^2, \tag{6a}$$

where $S_{i,0}$ is the sedimentation coefficient of species i at infinite dilution of all macromolecular solutes, and

$$\overline{S}_0 \equiv \sum_i \alpha_i S_{i,0}. \tag{6b}$$

Definition of μ_r, the r-th moment of the boundary-gradient curve. Remembering that n_c (equation 4a) is taken to be a measurable quantity, we define the rth moment of the gradient curve as

$$\mu_r \equiv \frac{\int_{x_0}^{x_w} x^r (\partial n_c/\partial x)\,\mathrm{d}x}{\int_{x_0}^{x_w} (\partial n_c/\partial x)\,\mathrm{d}x} \quad (r = 0,\ 1,\ 2,\ \ldots), \tag{7a}$$

and so, in terms of the individual concentration-gradient curves,

$$\mu_r = \frac{\sum_i R_i \int_{x_0}^{x_w} x^r (\partial c_i/\partial x)\,\mathrm{d}x}{\sum_i R_i c_i^\beta}, \tag{7b}$$

where we require that all $\partial c_i/\partial x = 0$ at x_0, the position of the meniscus, and at x_w, an arbitrary plane in the region of constant concentration ahead of the boundary (the region marked β in Fig. 1); c_i^β is the concentration of solute i in the β region. [It can be shown from equations (10) and (11) that, when S is a function of c only, there will be a region ahead of the boundary in which c does not vary with distance; cf. Trautman & Schumaker, 1954.]

The time derivative of μ_r. Equation (7b) may be differentiated by applying Leibnitz's rule [cf. Courant (1936) or Kaplan (1953, p. 217)] which requires that the indicated partial derivatives be continuous:

$$\frac{\mathrm{d}\mu_r}{\mathrm{d}t}\sum_i R_i c_i^\beta + \mu_r \sum_i R_i \frac{\mathrm{d}c_i^\beta}{\mathrm{d}t} = \sum_i R_i \int_{x_0}^{x_w} x^r \left[\frac{\partial}{\partial t}\left(\frac{\partial c_i}{\partial x}\right)_t\right]_x \mathrm{d}x. \tag{8}$$

The order of differentiation can be inverted, again provided that the partial derivatives are continuous:

$$\left[\frac{\partial}{\partial t}\left(\frac{\partial c_i}{\partial x}\right)_t\right]_x = \left[\frac{\partial}{\partial x}\left(\frac{\partial c_i}{\partial t}\right)_x\right]_t. \tag{9}$$

The continuity equation for transport in a sector-shaped cell is

$$\left(\frac{\partial c_i}{\partial t}\right)_x = -\frac{1}{A}\left(\frac{\partial A J_i}{\partial x}\right)_t, \tag{10}$$

where A, the cell area, is given by

$$A = (\text{const.})\ x, \tag{10a}$$

x being the distance from the centre of rotation. Equation (10) expresses simply the conservation of mass, stated in a differential form. The concentration, c, must be on a weight/volume scale and the flow, J (the amount transferred across unit area in unit time), must be expressed in corresponding units. Lamm's (1929) flow equation will be used, with the assumption that the diffusion and sedimentation coefficients are functions only of the various solute concentrations:

$$J_i = -D_i \frac{\partial c_i}{\partial x} + c_i S_i \omega^2 x. \tag{11}$$

In equation (11), D_i is the diffusion coefficient of i and ω is the angular speed of rotation.

After substitution of equations (9), (10) and (11) into equation (8), one may integrate by parts to obtain the following expression for the time derivative of μ_r:

$$\frac{\mathrm{d}\mu_r}{\mathrm{d}t} = 2\omega^2 \mu_r \sum_i R_i c_i^\beta S_i^\beta / n_c^\beta + (r)\,(r-2) \sum_i R_i \int_{x_0}^{x_w} x^{(r-2)}\, D_i (\partial c_i/\partial x)\, \mathrm{d}x / n_c^\beta + (r-2)\,\omega^2 \sum_i R_i \int_{x_0}^{x_w} x^r \left[\frac{\partial}{\partial x}(c_i S_i)\right]_t \mathrm{d}x / n_c^\beta, \tag{12}$$

where

$$n_c^\beta \equiv \sum_i R_i c_i^\beta. \tag{12a}$$

In order to bring equation (12) into a form suitable for integrating with respect to time, it is necessary to state the exact concentration dependence of S_i and D_i.

The dependence of S_i upon the concentrations of the various solutes is conveniently expressed by means of a Taylor's series (cf. Kaplan, 1953, p. 369). Since p, $\overline{S}_0$ and μ_r have all been defined in terms of the refractive increments, $R_j c_j$, rather than the concentrations c_j, the equations are handled more readily if S_i is related to $R_j c_j$ instead of to c_j.

$$S_i = S_{i,0} - \sum_{j=1}^{n} a_{ij} R_j c_j - \sum_{j=1}^{n} \sum_{k=1}^{n} a_{ijk} R_j c_j R_k c_k - \ldots, \tag{13}$$

where $S_{i,0}$ is the sedimentation coefficient of species i at infinite dilution of all n macromolecular species and the a_{ij} and a_{ijk} are partial derivatives evaluated at infinite dilution of all solutes:

$$a_{ij} \equiv -\left(\frac{\partial S_i}{\partial R_j c_j}\right)_{R_k c_k (k \neq j)}, \tag{13a}$$

$$a_{ijk} \equiv -\frac{1}{2}\left(\frac{\partial^2 S_i}{\partial R_j c_j \partial R_k c_k}\right)_{R_l c_l (l \neq j,k)}. \tag{13b}$$

In order to solve equation (12) we assume that all a_{ij} have the same value, a_1, and also that all a_{ijk} are the same (a_2):

$$S_i = S_{i,0} - a_1 n_c - a_2 n_c^2 - \ldots. \tag{13c}$$

As the final equation (20) for p shows, the dependence of D_i on concentration has little effect on the shape of the boundary. A linear relation of D_i to n_c is assumed here:

$$D_i = D_{i,0} - K n_c. \tag{13d}$$

An expression for $\mathrm{d}\sigma^2/\mathrm{d}t$. The definition of σ^2 is simply

$$\sigma^2 \equiv \mu_2 - (\mu_1)^2, \tag{14}$$

where μ_2 and μ_1 are defined in turn by equation (7a). The first moment, μ_1, has been denoted by $\overline{x}$ in this series of articles:

$$\mu_1 \equiv \overline{x}. \tag{14a}$$

Differentiation of equation (14) with respect to time gives

$$\frac{\mathrm{d}\sigma^2}{\mathrm{d}t} = \frac{\mathrm{d}\mu_2}{\mathrm{d}t} - 2\overline{x}\frac{\mathrm{d}\overline{x}}{\mathrm{d}t}. \tag{15}$$

Substitution of equations (13c) and (13d) into equation (12) enables one to evaluate $\mathrm{d}\mu_2/\mathrm{d}t$ and $\mathrm{d}\overline{x}/\mathrm{d}t$ with the result that

$$\frac{\mathrm{d}\sigma^2}{\mathrm{d}t} = 2\omega^2\sigma^2 \sum_i R_i c_i^\beta S_i^\beta / n_c^\beta + 2\overline{x} \sum_i R_i \int_{x_0}^{x_w} x^{-1} D_i (\partial c_i/\partial x)\, \mathrm{d}x / n_c^\beta + 2\omega^2 \overline{x} \sum_i R_i c_i^\beta S_{i,0} (\overline{x}_i - \overline{x}) / n_c^\beta - 2\omega^2 \overline{x} (2a_1 Q_1 + 3a_2 Q_2), \tag{16}$$

where

$$\overline{x}_i \equiv \int_{x_0}^{x_w} x (\partial c_i/\partial x)\, \mathrm{d}x / c_i^\beta, \tag{16a}$$

$$Q_1 \equiv \int_{x_0}^{x_w} (x - \overline{x})\, n_c (\partial n_c/\partial x)\, \mathrm{d}x / n_c^\beta, \tag{16b}$$

$$Q_2 \equiv \int_{x_0}^{x_w} (x - \overline{x})\, n_c^2 (\partial n_c/\partial x)\, \mathrm{d}x / n_c^\beta. \tag{16c}$$

Evaluation of the various summations in equation (16) (see Appendix) allows one to express $\mathrm{d}\sigma^2/\mathrm{d}t$ in such a way that it can be integrated with respect to time:

$$\frac{\mathrm{d}\sigma^2}{\mathrm{d}t} = 2\omega^2\sigma^2(\overline{S}^\dagger + \ldots) + 2\overline{D}_0[1 + 4\omega^2 t(\overline{S}_0 - \overline{S}_0') + \ldots] - K n_c^0 + \ldots + 2p^2\omega^4 x_0^2 t\{1 + (\omega^2 t)\,[-(\tfrac{3}{2})\,(q^3/p^2) + 2\overline{S}^\dagger] + \ldots\} + Q_1(-4\omega^2\overline{x}\,a_1 + 2K/\overline{x}) - 6\omega^2\overline{x}\,a_2 Q_2, \tag{17}$$

where

$$\overline{S}^\dagger \equiv \overline{S}_0 - a_1 n_c^0 - a_2 (n_c^0)^2, \tag{17a}$$

$$n_c^0 \equiv \sum_i R_i c_i^0, \tag{17b}$$

$$\overline{D}_0 \equiv \sum_i \alpha_i D_{i,0}, \tag{17c}$$

$$\overline{S}_0' \equiv \sum_i \alpha_i D_{i,0} S_{i,0} / \overline{D}_0, \tag{17d}$$

$$q^3 \equiv \sum_i \alpha_i (S_{i,0} - \overline{S}_0)^3. \tag{17e}$$

This equation can be compared with one previously derived for the case that S_i and D_i are independent of concentration (Baldwin, 1954*b*). When the expression for σ^2 given in that article (equation 23) is differentiated with respect to time and the result compared with the equation above, it may be seen that the two are identical once the terms of equation (17) in a_1, a_2 and K are set equal to zero.

An equation for σ^2. Since

$$\sigma^2 = \int_{t_c}^{t} \frac{\mathrm{d}\sigma^2}{\mathrm{d}t}\,\mathrm{d}t + \sigma_0^2, \tag{18}$$

where σ_0^2 is the value of σ^2 at the time t_0 of the first photograph, integration of equation (17) gives

$$\begin{aligned}\sigma^2 = \sigma_0^2 &+ 2\omega^2(\overline{S}^{\dagger}+\ldots)\int_{t_0}^{t}\sigma^2\mathrm{d}t \\ &+ 2\overline{D}_0[(t-t_0)+2\omega^2(\overline{S}_0-\overline{S}_0')(t^2-t_0^2)+\ldots] \\ &- Kn_c^0(t-t_0)+\ldots+p^2\omega^4x_0^2 \\ &\times\{(t^2-t_0^2)+\omega^2(t^3-t_0^2)[-(q^3/p^2)+(\tfrac{4}{3})(\overline{S}^{\dagger})]+\ldots\} \\ &- 2\omega^2\int_{t_0}^{t}\bar{x}(2a_1Q_1+3a_2Q_2)\,\mathrm{d}t + 2K\int_{t_0}^{t}(Q_1/\bar{x})\,\mathrm{d}t.\end{aligned} \tag{19}$$

The final equation for p, *in terms of measurable quantities*. By omitting from equation (19) the relatively minor terms (cf. Baldwin, 1954*b*) in $(\overline{S}_0-\overline{S}_0')$, q^3 and KQ_1, and by defining the new variables B and $\mathrm{F}(t)$, a simple equation is obtained for p, the standard deviation of the distribution of S:

$$p = [B/\omega^4x_0^2\,\mathrm{F}(t)]^{\frac{1}{3}}, \tag{20}$$

$$B \equiv \sigma^2 - \sigma_0^2 - 2\omega^2\overline{S}^{\dagger}\int_{t_0}^{t}\sigma^2\,\mathrm{d}t - 2(\overline{D}_0-\tfrac{1}{2}Kn_c^0)(t-t_0) + 2\omega^2\int_{t_0}^{t}\bar{x}(2a_1Q_1+3a_2Q_2)\,\mathrm{d}t, \tag{20a}$$

$$\mathrm{F}(t) \equiv (t^2-t_0^2)+(\tfrac{4}{3})(\omega^2\overline{S}^{\dagger})(t^3-t_0^3). \tag{20b}$$

The plot of B against $\mathrm{F}(t)$ is a straight line, with slope $p^2\omega^4x_0^2$, and is used to find p.

When it is not possible to make independent diffusion measurements, so that the value of $(\overline{D}_0-\frac{1}{2}Kn_c^0)$ is unknown, equation (19) may be used in another way. We define $\sigma^2_{\mathrm{corr.}}$ by

$$\sigma^2_{\mathrm{corr.}} \equiv \sigma^2 - 2\omega^2\overline{S}^{\dagger}\int_{t_0}^{t}\sigma^2\,\mathrm{d}t + 2\omega^2\int_{t_0}^{t}\bar{x}(2a_1Q_1+3a_2Q_2)\,\mathrm{d}t, \tag{21}$$

and plot $\sigma^2_{\mathrm{corr.}}$ against $(t-t_0)$. If the solute is homogeneous, the plot will be a straight line with slope $2(\overline{D}_0-\frac{1}{2}Kn_c^0)$. If the solute is heterogeneous, the plot will have upward curvature.

Comparison with the case of a rectangular cell and constant field

In view of the numerous terms in equation (17), one might inquire if the approach to this problem is unwieldy. The corresponding equation for the hypothetical case of a rectangular cell and constant field indicates that this is not the case. Equation (17) reduces, for this case, to

$$\begin{aligned}\mathrm{d}\sigma^2/\mathrm{d}t = {} & 2p^2\omega^4x_0^2\,t \\ & + 2(\overline{D}_0-\tfrac{1}{2}Kn_c^0) - 2(\omega^2x_0)\,(2a_1Q_1+3a_2Q_2),\end{aligned} \tag{22}$$

where the (constant) field strength has been denoted by ω^2x_0. The equation could hardly be simpler, so that one must judge the number of terms in equation (17) to be a consequence of the experimental requirements of a sector-shaped cell and field strength, ω^2x, proportional to x.

METHODS OF CALCULATION

In order to calculate p from a given sedimentation experiment, the following groups of quantities are required. (i) Those which are computed from each schlieren photograph by numerical integration: $\bar{x}$, σ^2, Q_1, Q_2 and n_c^0. (ii) Those which must be found from additional experiments: a_1, a_2 and $(\overline{D}_0-\frac{1}{2}Kn_c^0)$. (iii) Others. The angular speed of rotation, ω, and the time from the first photograph $(t-t_0)$ are found readily. Both t_0 and $\overline{S}^{\dagger}$ are found from equation (30), which relates the boundary position to time.

The schlieren curves produced by sedimentation of a protein and by the buffer in which it is dissolved are shown diagrammatically in Fig. 1*a* and *b* respectively. The latter schlieren curve (Fig. 1*b*) is termed the 'reference base line'; it is obtained under exactly the same conditions (the same cell, degree of filling, speed of rotation, time after reaching full speed and angle of the diagonal wire) as the former. The distance between two points in the cell, x_1 and x_2, is magnified on the schlieren curve by a factor F:

$$X_2 - X_1 = F(x_2 - x_1), \tag{23}$$

where X is the co-ordinate axis on the photographic plate which corresponds to the direction of sedimentation in the cell.

The other co-ordinate of the schlieren curve, Z, is linearly related to $\partial n/\partial x$:

$$Z = G\mathrm{ab}\cot\psi[\partial n/\partial x + \mathrm{f}(x)]. \tag{24}$$

In equation (24) $\mathrm{f}(x)$ expresses the prismatic deflexion of the schlieren curve caused by deformation of the cell windows: cf. Svedberg & Pedersen (1940, p. 268). (It is a function not only of x but of the particular cell, the degree of tightening of the guard rings, and the density and refractive index of the solution.) Denoting Z in the reference baseline experiment by Z_0, we have

$$Z_0 = G\mathrm{ab}\cot\psi[\partial n_0/\partial x + \mathrm{f}_0(x)]. \tag{24a}$$

If $\mathrm{f}(x)$ can be set equal to $\mathrm{f}_0(x)$ (cf. Ogston & Woods, 1954), then

$$Z - Z_0 \equiv Z_c = G\mathrm{ab}\cot\psi(\partial n_c/\partial x). \tag{24b}$$

Measurement of the cell thickness, a, the optical lever arm, b, and the magnification factors F and G, is discussed by Longsworth (1946) and Cecil & Ogston (1948). In equation (24) the angle of the diagonal wire, ψ, is measured from a line parallel to the slit image at the diagonal wire.

By means of a comparator, values of (Z, X) are read from the photographic plate at a fixed interval ΔX so chosen that there will be twenty or so readings taken in the boundary region. The stage of the comparator is free to travel in two directions, at right angles to each other. In order to align the photographic plate on the stage, the meniscus trace is set parallel to one axis of the comparator because this trace represents an infinite gradient of refractive index and is thus parallel to the Z axis. Since $\partial n_c/\partial x$ is expected to be zero in the region of constant concentration ahead of the boundary (the β region of Fig. 1), the origin for Z_c is found by superimposing the reference baseline (Fig. 1b) and the schlieren curve of Fig. 1a in the β region.

In view of the theoretical (Kegeles & Gosting, 1947) and practical uncertainties of measuring $\partial n_c/\partial x$ (approx. 1%), the method of numerical integration known as trapezoidal integration is sufficiently accurate to find $\bar{x}$, σ^2, Q_1 and Q_2 and n_c^0. Given a set of n values of y $(y_1, y_2, \ldots, y_n)$, where y is a continuous function of z and the values of y are spaced at the constant interval Δz, the integral $\int y\,dz$, evaluated between the limits of z at y_1 and y_n, is approximated by

$$\int y\,dz = \Delta z \left\{ \sum_{i=2}^{n-1} y_i + \tfrac{1}{2}(y_1 + y_n) \right\}. \qquad (25a)$$

If $y_1 = y_n = 0$, this reduces to

$$\int y\,dz = \Delta z \Sigma y_i. \qquad (25b)$$

The calculations are simplified by choosing an arbitrary origin X' from one of the set of readings (X, Z) and by writing $X - X'$ as $h\Delta X$. Thus h takes the values 1, 2, 3, ..., to the right of X' and -1, -2, -3, ..., to the left of X'. The position in the cell, x', corresponding to X' on the photographic plate is

$$x' = x_I + (X' - X_I)/F, \qquad (26)$$

where x_I is the distance from the centre of rotation to an edge of the index hole in the balance cell, and X_I marks this edge of the index trace on the photographic plate. Then the following equations are obtained from equations (25a) and (25b) for $\bar{x}$, σ^2, etc.:

$$\bar{x} = x' + \left(\frac{\Delta X}{F}\right) \frac{\Sigma h Z_c}{\Sigma Z_c}, \qquad (27a)$$

$$\sigma^2 = \left(\frac{\Delta X}{F}\right)^2 \frac{\Sigma h^2 Z_c}{\Sigma Z_c} - (x' - \bar{x})^2, \qquad (27b)$$

$$Q_1 = \left(\frac{\Delta X}{F}\right) \frac{\Sigma h n_c Z_c}{\Sigma Z_c} + \frac{(x' - \bar{x})}{2} n_c^\beta, \qquad (27c)$$

$$Q_2 = \left(\frac{\Delta X}{F}\right) \frac{\Sigma h n_c^2 Z_c}{\Sigma Z_c} + \frac{(x' - \bar{x})}{3} (n_c^\beta)^2, \qquad (27d)$$

$$n_c^\beta = \frac{\Delta X}{F G a b \cot \psi} \Sigma Z_c, \qquad (27e)$$

where the value of n_c in equations (27c) and (27d) for the jth set of readings of (X, Z) is given by

$$(n_c)_j = \frac{\Delta X}{F G a b \cot \psi} \left\{ \sum_{i=2}^{j-1} (Z_c)_i + \tfrac{1}{2}(Z_c)_1 + \tfrac{1}{2}(Z_c)_j \right\}. \qquad (27f)$$

In order to see how accurately σ^2, n_c^β and Q_1 are given by this kind of numerical integration with a limited set of $\partial n_c/\partial x$ values, they were calculated from tabulated values of the Gaussian function, or probability integral. It was assumed that values of $\partial n_c/\partial x$ less than 0·5% of the maximum gradient would not be detected experimentally, and so these were omitted from the calculations. Taking seventeen values of $\partial n_c/\partial x$ spaced throughout the

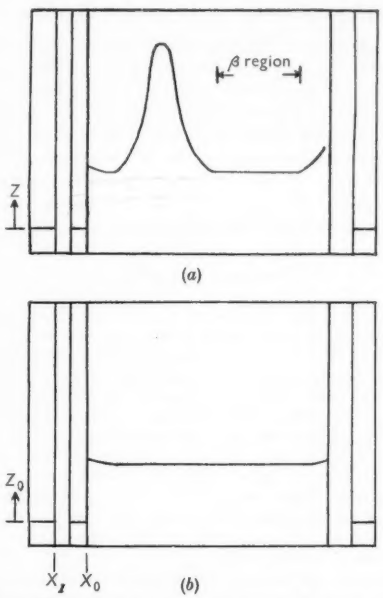

Fig. 1. Diagrams of the schlieren curves observed during sedimentation (a) of a protein solution and (b) of the buffer in which the protein is dissolved. Sedimentation is from left to right. The position of the meniscus trace is labelled X_0 and the edge of an index trace is marked X_I.

gradient curve (including the maximum gradient and eight values on each side of the maximum) gave values for σ^2, n_c^β and Q_1 which were 0·8, 0·06 and 0·4% too low respectively. (For a Gaussian curve, Q_1 is given by $\sigma n_c^\beta/2\sqrt{\pi}$.) Increasing the number of $\partial n_c/\partial x$ values to 35 reduced the error very little; the calculated values were then 0·7, 0·05 and 0·3% too low. At present, the uncertainty introduced by this use of numerical integration is not a limiting source of error.

In order to check the experimental accuracy of measurements of $\partial n_c/\partial x$, n_c^0 also is calculated from each photograph and then values from successive photographs compared. According to Trautman & Schumaker (1954)

$$n_c^0 = \int_{x_0}^{x_\infty} x^2(\partial n_c/\partial x)\,\mathrm{d}x/x_0^2, \qquad (28a)$$

and thus (cf. equation 7*a*)

$$n_c^0 = n_c^\beta(\mu_2/x_0^2) = n_c^\beta(\overline{x}^2+\sigma^2)/x_0^2, \qquad (28b)$$

where σ^2 is only of the order of 0·05% of $\overline{x}^2$. Another check on the accuracy of $\partial n_c/\partial x$ values is the comparison of values for n_c^0 found from the sedimentation experiment with $(n_c^0)_{\text{D.R.}}$, the value found by a differential refractometer.

To obtain $\overline{D}_0 - \frac{1}{2}Kn_c^0$, a free diffusion experiment is carried out in which the mean concentration is equal to half the initial concentration of the sedimentation experiment. Then this average diffusion coefficient is calculated from $\sigma^2/2t$, where the definition of σ^2 is still that given in equations (14) and (7) (cf. Gralén, 1944). To convert the diffusion coefficient to the temperature and buffer of the sedimentation experiment one may use the approximate relation (cf. Longsworth, 1954)

$$\eta D/T = \text{constant}, \qquad (29)$$

where η is the solvent viscosity and T is the absolute temperature.

The measurement of sedimentation coefficients is discussed in the next paper and will not be considered in detail here except to point out differences between the calculations for a homogeneous and for a heterogeneous system. Equation (A 10) of the appendix may be written (cf. Goldberg, 1953)

$$\ln(\mu_2/x_0^2)^{\frac{1}{2}} - (\omega^2 t)^2(\overline{S}^\dagger)\,[a_1 n_c^0 + 2a_2(n_c^0)^2 - (p^2/\overline{S}^\dagger)] + \ldots = \overline{S}^\dagger\omega^2 t. \qquad (30)$$

Thus to calculate $\overline{S}^\dagger$ from a given experiment, one needs preliminary estimates of a_1, a_2, p and t_0. Since the term in $(\omega^2 t)^2$ is usually not very large, these preliminary estimates need not be accurate. Then the left-hand side of equation (30) is plotted against $t-t_0$, the time from the first photograph; the slope gives $\overline{S}^\dagger\omega^2$ and the intercept, when the left-hand side = 0, gives $\int_0^{t_0}\omega^2\mathrm{d}t$. The value of t_0 required for equation (20*b*) is given by the ratio of this integral to the value of ω^2 at full speed. (Another way of plotting this kind of equation is described in the next paper.) The value of $\overline{S}^\dagger$ is converted to standard conditions by the approximate relation

$$S\eta/(1-\overline{v}\rho) = \text{constant}, \qquad (31)$$

where $\overline{v}$ is the solute's partial specific volume and ρ is the density of the solution. Finally the plot of $\overline{S}^\dagger$ against n_c^0 yields values for a_1 and a_2 and these are compared with the preliminary estimates used in calculating $\overline{S}^\dagger$, to determine whether recalculation is necessary.

When the optical constants a, b and G are unknown, the following procedure can be used to evaluate a_1Q_1 and a_2Q_2. From equations (27*c–f*) and (28*b*), $G\text{ab}Q_1$, $(G\text{ab})^2Q_2$ and $G\text{ab}n_c^0$ can be calculated. The plot of $\overline{S}_2$ against C^0, where C^0 is the initial total concentration,

$$C^0 = \Sigma c_i^0 \qquad (32)$$

yields $a_1\overline{R}$ and $a_2\overline{R}^2$, the definition of $\overline{R}$ being

$$\overline{R} \equiv n_c^0/C^0 \qquad (33)$$

(cf. equation 17*b*). Then

$$a_1Q_1 = (a_1\overline{R})\,(G\text{ab}Q_1)\,(C^0)/(G\text{ab}\,n_c^0), \qquad (34a)$$

$$a_2Q_2 = (a_2\overline{R}^2)\,[(G\text{ab})^2Q_2]\,[C^0/G\text{ab}\,n_c^0]^2, \qquad (34b)$$

Next $\int\sigma^2\,\mathrm{d}t$ and $\int\overline{x}(2a_1Q_1+3a_2Q_2)\,\mathrm{d}t$ are found by equation (25*a*) with Δt for Δz and, for y, σ^2 and $\overline{x}(2a_1Q_1+3a_2Q_2)$ respectively. At this point all the quantities required to calculate B and $\mathrm{F}(t)$ (equations 20*a* and *b*) are known. Note that equation (20*a*) calls for values of a_1, a_2, $\overline{S}^\dagger$ and $\overline{D}_0 - \frac{1}{2}Kn_c^0$ for the solvent and temperature of the sedimentation experiment. Finally p^2 is found from the plot of B against $\mathrm{F}(t)$ and then p is computed and converted to standard conditions by equation 31.

EXPERIMENTAL

Materials. Two samples of Armour crystalline bovine plasma albumin were investigated. Lot no. 10 522 was examined in a buffer containing 0·15 M-NaCl, 0·03 M total acetate (acetic acid and Na acetate), pH 4·34 (glass electrode, 20°). The buffer used in studying Lot no. R 370 295 A contained 0·10 M-KCl, 0·01 M acetic acid and 0·01 M-Na acetate; its pH (glass electrode, 25°) was 4·55. The sample of bovine β-lactoglobulin, prepared by the modified (Smithies, 1953) method of Cecil & Ogston (1949), was the gift of Dr J. M. A. Tilley. This sample was studied in the same buffer (0·1 M-NaCl, 0·1 M-Na acetate, 0·04 M acetic acid) used by Cecil & Ogston (1949) in their study of the sedimentation and diffusion of β-lactoglobulin.

Methods. Calibration of the Spinco ultracentrifuge, used in studying albumin sample no. R 370 295 A, is described in the following article. The Oxford oil-turbine ultracentrifuge, used in studying the lactoglobulin and the other albumin sample, also has been described elsewhere: Cecil & Ogston (1948) and Baldwin (1953*b*). The diagonal bar-schlieren system was focused under running conditions. A mono-

chromator isolates the green line of a 250 w, box-type air-cooled mercury lamp. The rotor speed is measured by an apparatus designed and built by Dr R. Cecil which records the revolutions of a phonic motor. Since one shaft of the rotor is magnetized, there is a current generated as the rotor turns and this current is amplified and used to drive the phonic motor. The rotor thermocouple was calibrated with diphenyl ether in the manner described by Cecil & Ogston (1948).

Refractive increments were measured, after dialysis, with the differential refractometer of Cecil & Ogston (1951); a diffraction pattern produced by a single slit was used to measure the deflections.

RESULTS AND DISCUSSION

β-Lactoglobulin. No measurable heterogeneity was found. The values of B, and hence of p (equation 20), did not differ significantly from zero; the largest value was only 1% of $(\sigma^2-\sigma_0^2)$. Consequently the plot of $\sigma^2_{corr.}$ (equation 21) against $(t-t_0)$ should be a straight line with slope $2(\bar{D}_0-\frac{1}{2}Kn_c^0)$. This plot is shown in Fig. 2. The value of D computed from its slope may be compared with the diffusion measurements of Cecil & Ogston (1949); interpolation of their data gives a value for $D_{20,w}$ of $7{\cdot}7_8\times10^{-7}$ cm.^{2} sec.$^{-1}$ at a mean concentration of 0·4 g./100 ml. From the slope of Fig. 2 a value for $D_{20,w}$ of $7{\cdot}7_6\times10^{-7}$ cm.^{2} sec.$^{-1}$ is obtained when equation (29) is used to convert the value of D from the temperature of the sedimentation experiment (28·2°) to that quoted by Cecil & Ogston. This agreement is somewhat fortuitous, since the experimental uncertainty of measuring D from a sedimentation experiment is about 5% (cf. the next paper).

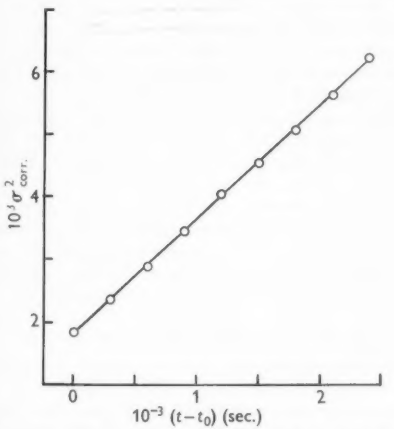

Fig. 2. Plot of $\sigma^2_{corr.}$ (equation 21) against the time from the first photograph, for a sample of lactoglobulin. When a solute displays no heterogeneity in sedimentation behaviour, this plot is a straight line with slope equal to twice the diffusion coefficient.

It would be interesting to know whether this sample contained one or both of the components observed by Aschaffenburg & Drewry (1955) on filter-paper electrophoresis. Unfortunately, none of this sample remains. In the light of the work of Ogston & Tilley (1955) and of Aschaffenburg & Drewry (1955), it is to be expected that different samples of β-lactoglobulin will show different behaviour. Cecil & Ogston (1949) found evidence for a rapidly diffusing impurity in certain β-lactoglobulin samples, and Creeth (personal communication) has also found this, in an amount which should be detectable in sedimentation experiments. If one takes a value of B equal to 5% of

$$2(\bar{D}_0-\tfrac{1}{2}Kn_c^0)\,(t-t_0)$$

as the least significant evidence of heterogeneity in sedimentation behaviour, then the least measurable value of p for this sedimentation experiment is computed to be 5% of $\bar{S}_0$. If there were two components present in equal amount, they would have to differ in S_0 by 10% of $\bar{S}_0$ or less (cf. equation 35*b*) with this value of p.

In deriving the theory given here, chemically reacting systems were excluded. Consequently it is important to note that Ogston & Tilley (1955) found that β-lactoglobulin associates at low temperatures. However, they concluded that association is negligible above 20°.

In general, the boundary-gradient curves produced by sedimentation of a homogeneous protein will not be Gaussian in shape because of the dependence of S on c (Fujita, 1956). However, the concentration dependence shown by β-lactoglobulin is so slight (Cecil & Ogston, 1949) that at the concentration studied here ($c^0=0{\cdot}78$ g./100 ml.) the boundary-gradient curves were found to be Gaussian. The plot of $\log \partial n_c/\partial x$ against $(x-x_H)^2$ was linear on both sides of x_H (the position of the maximum gradient) and values of σ calculated by equation (27*b*) agreed with those from the equation for a Gaussian curve within experimental error. (For a Gaussian curve, $\sigma=A/H\sqrt{2\pi}$, where A is the area and H the maximum height of the boundary-gradient curve.) The greatest difference between the two values of σ, in six photographs, was 2·1%; the mean difference was 1·2%. Consequently values of σ were taken from A and H, since these are more precise than the values of σ calculated by (27*b*), and also Q_1 was calculated from the equation for a Gaussian curve ($Q_1=\sigma n_c^\beta/2\sqrt{\pi}$). The value for $a_{1(25,w)}$ was calculated to be $0{\cdot}055\times10^{-13}/\bar{R}$, from the data of Cecil & Ogston (1949). From the results of Perlmann & Longsworth (1948), $1{\cdot}86\times10^{-3}$ was computed for $\bar{R}$ at 25° and the mercury green line. The areas under six successive boundary-gradient

curves were used to calculate n_c^0 (equation 28*b*) for comparison with $(n_c^0)_{D.R.}$, the value found with a differential refractometer (D.R.). The average value of the ratio $(n_c^0)_{D.R.}/(n_c^0)_{U.C.}$ (U.C., ultracentrifuge) was found to be 0·999, with a standard error of 0·009.

Bovine plasma albumin. Different values of p (Table 1) were found for the two different samples of albumin, although both samples are crystalline and were obtained from Armour and Co. For sample 10 522,

$$p_{25,w} = 1{\cdot}0_8 \pm 0{\cdot}09\,\text{s},$$

while for sample R 370 295A

$$p_{25,w} = 0{\cdot}69 \pm 0{\cdot}02\,\text{s}$$

(s = Svedberg units). However, sample 10 522 was studied at a somewhat lower pH (4·34) than that used with R 370 295A (pH 4·55) and dependence on pH of various physical properties of albumin has been reported by several workers: cf. Tanford, Buzzell, Rands & Swanson (1955) and Harrington, Johnson & Ottewill (1956).

In view of these different values for p, it is of interest to compare other properties of the two samples which are related to their heterogeneity. The ratio of the mean sedimentation coefficient to that of the main component (Table 2) is not significantly different for the two samples. In the next paper, measurements are discussed which allow one to estimate the amount of a faster-sedimenting component in both samples; the fractional amount, α_2, of this component was not found to be significantly different in the two samples. By use of Fujita's (1956) solution to the continuity equation, the homogeneity of the main component was investigated for both samples and is reported in the next paper. Here again the two samples differed: the main component of R 370 295A showed no measurable heterogeneity, whereas that of 10 522 did show heterogeneity.

In order to see how well the values of p and $\overline{S}_0/S_{1,0}$ can be interpreted in terms of only two solutes (1 and 2), equations were developed which relate each of these quantities to

$$(S_{2,0}-S_{1,0})/S_{1,0}.$$

$$\frac{S_{2,0}-S_{1,0}}{S_{1,0}} = \frac{(\overline{S}_0/S_{1,0})-1}{\alpha_2}, \qquad (35a)$$

$$\frac{S_{2,0}-S_{1,0}}{S_{1,0}} = \frac{p}{S_{1,0}(\alpha_1\alpha_2)^{\frac{1}{2}}}. \qquad (35b)$$

With sample R 370 295A, both of these equations give the same value for $(S_{2,0}-S_{1,0})/S_{1,0}$ within experimental error. Taking $\overline{S}_0/S_{1,0}$ as $1{\cdot}040 \pm 0{\cdot}005$ and α_2 as $0{\cdot}12 \pm 0{\cdot}02$ gives

$$(S_{2,0}-S_{1,0})/S_{1,0} = 0{\cdot}33 \pm 0{\cdot}10.$$

Taking p as $0{\cdot}69 \pm 0{\cdot}02$ s and $S_{1,0}$ as 5·01 s gives

$$(S_{2,0}-S_{1,0})/S_{1,0} = 0{\cdot}42 \pm 0{\cdot}05.$$

Taken together, these lead to an estimate for $(S_{2,0})_{25,w}$ of $7{\cdot}0 \pm 0{\cdot}3$ s. For sample 10 522, the estimate of $\overline{S}_0/S_{1,0}$ is much more uncertain so that it is not possible to say whether the higher value of p can be explained by a higher value of $S_{2,0}$ or by heterogeneity of the leading component. It can be shown from equation (6*a*) that the heterogeneity of the main component, which is discussed in the next paper, contributes only 5% to the overall value of p. The heterogeneity in diffusion behaviour found by Gosting (reported by Baldwin *et al.* 1955*b*) with different samples of Armour crystalline bovine albumin could be interpreted in terms of only two solutes.

In Expts. A 1, A 2, A 3 and B 1, S was treated as a linear function of n_c with a_1 (equation 13*c*) equal to 0·057 $\overline{S}_0/\overline{R}$; for the Expt. B 2 at the higher concentration, $a_1 = 0{\cdot}054\overline{S}_0/\overline{R}$ and $a_2 = 0{\cdot}004\overline{S}_0/\overline{R}$. These values are taken from the dependence on concentration of the sedimentation coefficient of the main component (see next paper). From the results of Perlmann & Longsworth (1948) one can compute a value of $1{\cdot}92 \times 10^{-3}$ for $\overline{R}$ at 25°, 0·1M-NaCl and the mercury green line. The value taken for $\overline{D}_{25,w}$ ($6{\cdot}73 \times 10^{-7}$ cm.^{2} sec.$^{-1}$) is that found by Gosting (Baldwin *et al.* 1955*b*) for sample R 370 295A at a mean concentration of 0·515 g./100 ml. According to Creeth (1952), the dependence of D on c is very small (at pH 6·8). The temperatures of the sedimentation experiments were as follows: A 1, 26·8°; A 2, 27·0°; A 3, 27·3°; B 1, 24·5°; B 2, 25·1°.

Table 1. *Heterogeneity of two samples of crystalline bovine albumin*

Expt. no.[a]	$C^{0\,b}$	$p_{25,w}$ (±S.D.) (s)[c]
A 1	0·61	1·13±0·03
A 2	0·61	1·09±0·01
A 3	0·61	0·96±0·07
B 1	0·67	0·68±0·02
B 2	1·35	0·71±0·02

[a] Expts. A 1, A 2 and A 3 were carried out with sample no. 10 522 and with an oil-turbine ultracentrifuge. In Expts. B 1 and B 2, sample no. R 370 295A was studied in a Spinco ultracentrifuge.

[b] Initial concentration of the experiment in g./100 ml.

[c] The standard deviation of the sample's distribution of sedimentation coefficient, followed by the standard error of measurement, in Svedberg units.

Methods of checking the calculation of p

In computing p the first problem is to check on the measurements of $\partial n_c/\partial x$. The results in Table 2 show that these values of $\partial n_c/\partial x$ are reasonably precise, as judged by the agreement of values for n_c^0 from successive photographs. The solutions used for the sedimentation experiments had been dialysed and a differential refractometer (Cecil & Ogston, 1951) was used to measure the difference in

refractive index between solvent and solution. Table 2 shows that the values obtained with the differential refractometer agree with the values computed from the areas under the schlieren diagrams of the sedimentation experiments. This agreement means that the method of placing base lines is satisfactory provided that one can assume that (i) there are no large aggregates in the sample which sediment immediately to the bottom of the cell and do not contribute to the boundary curve, and (ii) that any differences in concentration of buffer constituents on the two sides of the dialysis membrane also occur on the two sides of the sedimenting boundary.

A small error in positioning the base line on the Z axis has a relatively large effect on the value for σ^2. This is shown in Table 3, where the values of σ^2 have been recalculated (and then denoted by σ'^2) after shifting the position of the base line on the Z axis enough to make the value of n_c^0 from each photograph agree with the value found by the differential refractometer, $(n_c^0)_{D.R.}$. This means that the variability of values for n_c^0 from successive schlieren photographs should be a fairly good guide to the reliability of the experiment. If these values do not show reasonable agreement, it will prove more profitable to find out why than to proceed further in computing p.

The standard error of a value for p is a fairly good guide to the reproducibility of the experiment, as the results of A 1, A 2 and A 3 show (Table 1). These experiments were carried out with the same initial solution of bovine albumin in order to test this. The values for p are reproducible within twice the standard error or less. [Mather (1946) discusses computation of the standard error when the slope of a straight line is being found.]

The contribution to B of the term involving the dependence of S on c can be varied by varying the initial concentration of the experiment. Thus in Expt. B 1, where $C^0 = 0{\cdot}67$ g./100 ml., the final value of the integral involving a_1 and a_2 is 26 % of the corresponding value of B (cf. Table 4). In B 2, where $C^0 = 1{\cdot}35$ g./100 ml., this integral is 44 % of the final value of B. The values of p from the two experiments agree within 4 %, or roughly the sum of their standard errors. In the previous theory for computing p (Williams *et al.* 1952; Baldwin, 1954*b*),

Table 2. *Other data from the experiments with albumin*

Expt. no.	$(n_c^0)_{D.R.}/(n_c^0)_{U.C.}$ ($\pm$S.D.)[a]	$\bar{S}_0/S_{1,0}$ ($\pm$S.D.)[b]
A 1	0·985$\pm$0·010	1·047$\pm$0·012
A 2	1·003$\pm$0·010	1·047$\pm$0·012
A 3	0·986$\pm$0·020	1·017$\pm$0·016
B 1	1$\pm$0·012[c]	1·038$\pm$0·005
B 2	1$\pm$0·006[c]	1·041$\pm$0·005

[a] Refractive increment of the albumin in the initial solution, as measured by a differential refractometer, divided by the mean value calculated from the areas under the boundary-gradient curves of the ultracentrifuge experiment. The standard error gives the variation of $(n_c^0)_{U.C.}$ values about the mean.

[b] Ratio of the mean sedimentation coefficient to that of the main component, at infinite dilution.

[c] The value of $(n_c^0)_{D.R.}$ was not determined; the standard error of $(n_0^0)_{U.C.}$ is given.

Table 3. *Effect of an error in positioning the reference base line on values for σ^2*

Photo no.[a]	$(n_c^0)_{D.R.}/(n_c^0)_{U.C.}$[b]	σ'^2/σ^2[c]
1	0·989	0·999
2	0·960	0·850
3	0·977	0·900
4	0·992	0·966
5	1·014	1·053

[a] Expt. A 3.

[b] Ratio of values for the refractive increment of the protein in the initial solution as found with a differential refractometer and from the area of the boundary-gradient curve of the ultracentrifugal experiment.

[c] Ratio of the second moment about the mean of the boundary-gradient curve after (σ'^2) and before (σ^2) adding a constant amount to each value of $\partial n_c/\partial x$ such that $(n_c^0)_{U.C.}$ then agrees exactly with $(n_c^0)_{D.R.}$.

Table 4. *An example of the relative importance of the various quantities from which p is computed*

Photo no.[a]	$\sigma^2 - \sigma_0^2$	$2\omega^2\bar{S}\dagger\int_{t_0}^{t}\sigma^2\,dt$	$2\bar{D}(t-t_0)$	$4\omega^2 a_1\int_{t_0}^{t}\bar{x}Q_1\,dt$[b]	B[c]
1	0	0	0	0	0
2	0·95	0·05	0·66	0·12	0·36
3	1·94	0·11	1·31	0·25	0·77
4	2·88	0·20	1·96	0·41	1·13
5	4·54	0·30	2·62	0·57	2·18
6	5·83	0·44	3·27	0·76	2·88

[a] Expt. B 1: BPA, lot no. R 370 295 A, $C^0 = 0{\cdot}67$ g./100 ml. All values have been multiplied by 10^3 and have units of cm.^{2}.

[b] In general, this integral contains a term in a_2, but $a_2 = 0$ in this case.

[c] B is defined by equation 20*a* as $(\sigma^2 - \sigma_0^2) - 2\omega^2\bar{S}\dagger\int_{t_0}^{t}\sigma^2\,dt - 2\bar{D}(t-t_0) + 2\omega^2\int_{t_0}^{t}\bar{x}(2a_1Q_1 + 3a_2Q_2)\,dt$.

[d] The plot of B against $F(t)$ (defined by equation (20*b*), is a straight line with slope $p^2\omega^4x_0^2$, p being the standard deviation of the distribution of sedimentation coefficient.

the dependence of S on c was not considered. The effect of omitting this factor may be seen in Fig. 3, where values of B have been computed by the present theory and also by the older theory, for Expt. B 2. It may be seen that the dependence of S on c is a significant factor in the calculation of p.

Applicability of the theory

In using this method to find p, the following considerations should be kept in mind. (i) No account has been taken of any dependence on pressure of S, D and R. This is not likely to be a serious source of error for aqueous solutions but might be when organic solvents are used. (ii) For systems which are both strongly concentration dependent and markedly heterogeneous, the representation of the dependence of S_i on n_c in equation (13c) will be inadequate when the initial concentration is more than a certain value. For the purpose of these calculations, equation (13c) will be considered satisfactory so long as any error introduced into B by the use of this equation is less than the experimental uncertainty of σ^2. Provided that $\overline{S}^{\dagger}/\overline{S}_0 > 0{\cdot}9$, this should not be a serious source of error. And, of course, it is not a source of error for a homogeneous system. (iii) If chemically reacting systems were to be considered, additional terms would have to be added to the continuity equation (10). (iv) The flow equation (11) does not include transport by convection, and it has been shown by Harrington & Schachman (1953) (see also Trautman, Schumaker, Harrington & Schachman, 1954) that this occurs in systems of two solutes when the boundaries separate completely from one another. (In the experiments reported here with bovine albumin, one continuous boundary-gradient curve was observed.) Also a more complicated flow equation would be required if there were significant transport caused by an electrical potential gradient (cf. Svedberg & Pedersen, 1940, p. 23). (v) The requirement that all $\partial c_i/\partial x = 0$ at x_0 (e.g. equations 7b and 12) excludes systems where S is so small, or D so large, that the boundary-gradient curve does not resolve from the meniscus.

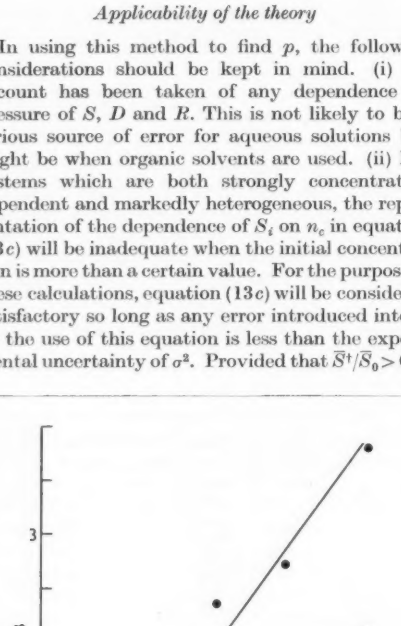

Fig. 3. Plot of B against $F(t)$ for sample R 370 295 A of crystalline bovine plasma albumin at an initial concentration of 1·35 g./100 ml. The points shown by filled circles have been calculated by equation (20); the open circles show the same data calculated by the older theory, without correction for the dependence of sedimentation coefficients on concentration. The slope of this plot (through the filled circles) is $p^2\omega^4 x_0^2$, p being the standard deviation of the sample's distribution of sedimentation coefficient.

On the other hand, the following factors have been taken into account: (i) heterogeneity in S, D and R; (ii) dependence on total concentration of S and D; (iii) the fact that the rotor does not achieve full speed instantaneously and also the initial period of restricted diffusion. Equations (17) and (19) apply once the boundary region has resolved from the meniscus, regardless of previous history. In computing B (equation 20a) it is not necessary to know the time from the beginning of diffusion but only the time $(t-t_0)$ from the first photograph. In computing $\mathrm{F}(t)$ (equation 20b) it is necessary to know the integral $\int_0^{t_0} \omega^2\,\mathrm{d}t$ as well, but this is readily obtained by use of equation (30).

Use of moments contrasted with solution of the continuity equation

In general, solution of the continuity equation (10) to give the shape of the boundary-gradient curves gives more information than does an equation for the moments of these curves. However, there are formidable mathematical difficulties in the way of solving the continuity equation for the shape of the boundary-gradient curve for the case presented here, in which the distribution of S is left unspecified and the flow equation for an individual solute includes a dependence of S on total concentration. The case of only two such concentration-dependent solutes has not yet been solved and the solution for a single solute (Fujita, 1956) is a major achievement.

Since Fujita's equation is limited, strictly, to the case of only one solute, it can be used to detect heterogeneity but not to measure it. Consequently it is most useful, from the standpoint of studying

heterogeneity, with systems which are on the borderline of detectable heterogeneity. With his equation, one can make use of the area and maximum height of the boundary-gradient curves and these can be measured more accurately than can σ. Also, the calculations required for use of Fujita's theory are less laborious than the ones required here. In the next paper, it was found possible to obtain some information about the homogeneity of a main component, when a small amount of another component is present. The theory given here cannot cope with a task such as this.

The chief use envisaged for this theory is in characterizing the heterogeneity of systems which are not sufficiently heterogeneous, or which diffuse too rapidly, for the distribution of S to be obtained by extrapolation to infinite time (cf. Baldwin, 1954*b*). Also, for systems in which $g(S)$ can be found by extrapolation to infinite time, it will be a valuable check on the accuracy of the extrapolated distribution to compute p both by this method and from $g(S)$ itself.

SUMMARY

1. A theory is developed for computing p, the standard deviation of the distribution of sedimentation coefficient in a sample containing macromolecular solutes. No assumption as to the form of the distribution is required.

2. The theory takes into account dependence of the sedimentation and diffusion coefficients upon the total concentration.

3. A sample of bovine β-lactoglobulin, prepared by the modified method of Cecil & Ogston (1948), showed no measurable heterogeneity in sedimentation behaviour. The resolving power of the method is sufficient to detect a value of p equal to 5 % of the mean sedimentation coefficient, despite present limitations on the accuracy of measuring boundary-gradient curves.

4. Two different samples of Armour crystalline bovine plasma albumin were found to have different values of p.

5. An equation is derived also for computing the mean sedimentation coefficient of a mixture, when the individual sedimentation coefficients depend on the total concentration.

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APPENDIX

Evaluation by expansion into series of the terms in equation (16). In order to find expressions for c_i^β and S_i^β, the concentration and sedimentation coefficient of i in the uniform region ahead of the boundary, the following procedure may be used (cf. Alberty, 1954). Let

$$S_i^\beta = S_i^\dagger + bt + \ldots, \qquad \text{(A 1)}$$

where $$S_i^\dagger \equiv S_{i,0} - a_1 n_c^0 - a_2 (n_c^0)^2 \qquad \text{(A 1}a\text{)}$$

and b is to be determined. Since all $\partial c_i/\partial x = 0$ in this region, substitution of equation (11) into (10) gives

$$\frac{\mathrm{d}c_i^\beta}{\mathrm{d}t} = -2c_i^\beta S_i^\beta \omega^2. \qquad \text{(A 2)}$$

Integration of equation (A 2), after substituting equation (A 1), yields

$$c_i^\beta = c_i^\dagger \exp(-2\omega^2 S_i^\dagger t + \ldots), \qquad \text{(A 3)}$$

or $$c_i^\beta = c_i^\dagger (1 - 2\omega^2 S_i^\dagger t + \ldots). \qquad \text{(A 3}a\text{)}$$

Thus
$$n_c^\beta = \sum_i R_i c_i^\dagger (1 - 2\omega^2 S_i^\dagger t + \ldots)$$
$$= n_c^\dagger (1 - 2\omega^2 \overline{S}^\dagger t + \ldots). \qquad \text{(A 4)}$$

[For the definition of $\overline{S}^\dagger$ and other symbols in this section, see equations (16) and (17).]

Substitution of equation (A 4) into equation (13*c*) and comparison of the resulting equation with (A 1) shows that

$$b = 2\omega^2 \overline{S}^\dagger [a_1 n_c^0 + 2a_2 (n_c^0)^2]. \qquad \text{(A 5)}$$

Thus, using equation (A 1) for S_i^β and (A 3*a*) for c_i^β, one finds that the first term on the right-hand side of equation (16) is

$$\sum_i R_i c_i^\beta S_i^\beta / n_c^\beta = \overline{S}^\dagger \{1 + 2(\omega^2 t)\,[a_1 n_c^0 + 2a_2 (n_c^0)^2 - (p^2/\overline{S}^\dagger)] + \ldots\}. \qquad \text{(A 6)}$$

Turning to the next term in equation (16), we first expand $(1/x)$ as

$$\frac{1}{x} = \frac{1}{\bar{x}}\left[1 - \left(\frac{x - \bar{x}}{\bar{x}}\right) + \ldots\right], \qquad \text{(A 7)}$$

and so

$$2\bar{x} \sum_i R_i \int_{x_0}^{x_w} x^{-1} D_i (\partial c_i/\partial x)\, \mathrm{d}x / n_c^\beta = 2 \sum_i R_i D_{i,0} c_i^\beta / n_c^\beta - K n_c^\beta - 2 \sum_i R_i D_{i,0} c_i^\beta (\bar{x}_i - \bar{x})/\bar{x} n_c^\beta + 2KQ_1/\bar{x} + \ldots. \qquad \text{(A 8)}$$

To obtain an expression for $(\bar{x}_i - \bar{x})$, one may write (cf. equation 14) that

$$\bar{x} = (\mu_2)^{\frac{1}{2}} [1 - (\sigma^2/2\mu_2) + \ldots]. \qquad \text{(A 9)}$$

Substitution of equation (A 6) into equation (12) for $r=2$ yields, after integration,

$$\mu_2 = x_0^2 \exp.\ \bar{S}^\dagger\{2\omega^2 t + 2(\omega^2 t)^2\ [a_1 n_c^0 + 2a_2(n_c^0)^2 - (p^2/\bar{S}^\dagger)] + \ldots\}. \quad \text{(A 10)}$$

Provided that the concentration-dependence terms are small enough for σ^2 to be approximated in equation (A 9) by the first terms of the non-concentration-dependent case (Williams *et al.* 1952; Baldwin, 1954*b*), one obtains for equation (A 9)

$$\bar{x} = [x_0 \exp S^\dagger\{\omega^2 t + (\omega^2 t)^2\ [a_1 n_c^0 + 2a_2(n_c^0)^2 - (p^2/\bar{S}^\dagger)] + \ldots\}] - x_0(p\omega^2 t)^2\ (1+\ldots)/2 - \bar{D}_0 t(1+\ldots)/x_0. \quad \text{(A 11)}$$

Similarly,

$$\bar{x}_i = \{x_0 \exp\ [\bar{S}_i^\dagger\ \omega^2 t + \tfrac{1}{2}\ b\omega^2 t^2 + \ldots]\} - D_{i,0} t(1+\ldots)/x_0. \quad \text{(A 12)}$$

Thus

$$(\bar{x}_i - \bar{x}) = \{x_0 \exp\ (\bar{S}^\dagger \omega^2 t)\}\ \{(S_{i,0} - \bar{S}_0)\ (\omega^2 t) + (\omega^2 t)^2[\tfrac{3}{2} p^2 + \tfrac{1}{2}(S_{i,0} - \bar{S}_0)^2] + \ldots\} + (\bar{D}_0 - D_{i,0})\ (t/x_0) + \ldots. \quad \text{(A 13)}$$

With this result, one can now evaluate equation (A 8):

$$2\bar{x} \sum_i R_i \int_{x_0}^{x_w} x^{-1} D_i(\partial c_i/\partial x)\ \mathrm{d}x/n_c^\beta = 2\bar{D}_0[1 + 3(\omega^2 t)\ (\bar{S}_0 - \bar{S}_0') + \ldots] - Kn_c^0 + 2KQ_1/\bar{x} + \ldots \quad \text{(A 14)}$$

The information required to evaluate the third term on the right-hand side of equation (16) is also now available:

$$2\omega^2 \bar{x} \sum_i R_i S_{i,0} c_i^\beta (\bar{x}_i - \bar{x})/n_c^\beta = 2p^2\omega^4 x_0^2 t \times \{1 + (\omega^2 t)\ [2\bar{S}^\dagger - (3/2)\ (q^2/p^2)] + \ldots\} + 2\bar{D}_0(\omega^2 t)\ (\bar{S}_0 - \bar{S}_0')\ (1+\ldots). \quad \text{(A 15)}$$

Substitution of equations (A 6), (A 14) and (A 15) into equation (16) yields equation (17).

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Boundary Spreading in Sedimentation-Velocity Experiments

5. MEASUREMENT OF THE DIFFUSION COEFFICIENT OF BOVINE ALBUMIN BY FUJITA'S EQUATION

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In sedimentation-velocity experiments, a boundary is formed as the solute sediments away from an air–liquid meniscus. The rate of movement of this boundary gives the solute's sedimentation coefficient (S). In general, the boundary is not perfectly sharp; hence it is customary to speak of the boundary-gradient curve, the graph (after subtraction of a reference base-line) of refractive index gradient against distance in the boundary region. Direct measurement of this curve may be made with the schlieren optical system (Philpot, 1938); cf. Fig. 1. The shape of the boundary-gradient curve is determined primarily by the solute's diffusion coefficient (D), its heterogeneity with respect to sedimentation behaviour, and its dependence of sedimentation coefficient upon concentration (c).

Recently, Fujita (1956) obtained an equation for the boundary-gradient curves observed when there is a single homogeneous solute with D constant but with S a linear function of c. Provided that an experimental system obeys the conditions assumed in deriving this equation, it is possible to measure D from the area and the maximum height of the boundary-gradient curve. In the past several attempts have been made to measure D from the boundary-gradient curves of sedimentation-velocity experiments by means of Faxén's equation (Faxén, 1929), which does not take account of the dependence of S on c. A procedure for doing this is given by Svedberg & Pedersen (1940, p. 286). It is now apparent that the omission of concentration dependence from Faxén's equation renders it inadequate for most experimental cases, except at concentrations far more dilute than those customarily used. One purpose of this article is to emphasize this fact.

Another purpose of this article is to illustrate a more general procedure for the measurement of diffusion coefficients from sedimentation-velocity experiments. Although values obtained in this way are less precise and more subject to error than those of conventional diffusion measurements, it is sometimes very useful to be able to measure the diffusion coefficient of a substance of which only 10 mg. can be obtained. At the same time, this measurement of D constitutes a sensitive test of the homogeneity of the solute since there are significant differences in the boundary-gradient curves of a homogeneous and of a heterogeneous substance. Also, by comparing the values of D obtained from sedimentation experiments and from conventional diffusion experiments, it is possible to check the validity of boundary-spreading measurements in sedimentation.

Bovine albumin was chosen for this study because accurate diffusion studies (Gosting, 1955) had been made of this sample and because its values of S and D are in the range where the theory applies reasonably well.

EXPERIMENTAL

Materials. Armour crystalline bovine plasma albumin, lot no. R370295A, was dissolved in a buffer of pH 4·55 (glass electrode, 25°), containing 0·10 M-KCl, 0·01 M acetic acid and 0·01 M-Na acetate. On the assumption that the viscosity and density increments of the buffer constituents are additive, this buffer was computed to have a density at 25° of 1·0024 and a viscosity relative to water of 1·005 (data from Svedberg & Pedersen, 1940, and Jones & Talley, 1933). Sedimentation coefficients were converted to 25° and the viscosity and density of water by the approximate relation $s\eta/(1-\bar{v}\rho) = \text{constant}$, where η is the solvent viscosity, ρ is the solution density and $\bar{v}$ is the solute's partial specific volume. The value of $\bar{v}$ used was 0·734 ml./g. (Dayhoff, Perlmann & MacInnes, 1952). According to the electrophoretic studies of Longsworth & Jacobsen (1949), the albumin should be very nearly isoelectric in this buffer. Concentrations were measured by the optical density at 280 mμ., by using a value for $E_{1\%}^{1\,\text{cm.}}$ cm. of 6·60 (Tanford & Roberts, 1952). Since the samples were prepared by weight dilution, an additional check was made of their relative concentrations.

Some experiments of a few years back, which were made with another sample of Armour crystalline bovine albumin (lot no. 10 522) at pH 4·34 using the Oxford oil-turbine ultracentrifuge, were recalculated by methods discussed in this article. Details of these experiments are given in the preceding paper.

Temperature measurement. Sedimentation was observed by means of a Spinco Model E ultracentrifuge equipped with the company's new temperature-control system which enables one to maintain constant temperature during an experiment within $\pm 0{\cdot}02°$. The temperature control and measurement (or 'RTIC') unit maintains a set resistance in an electrical circuit which includes the rotor. A thermistor

with a high resistance (approx. 13000Ω) and a temperature coefficient of −4% per degree is embedded in the base of the rotor. At the lower end of the rotor, electrical contact is made through a tungsten point dipping into mercury and, at the upper end, by a disk surrounded with mercury. The rotor chamber is refrigerated continuously and the bridge-circuit balance determines whether a wire radiation heater is on or off.

In order to correlate the temperature of the rotor with the dial reading of the RTIC unit, one takes the rotor outside its chamber and places it on a stand with electrical connexions to the RTIC circuit. Then the RTIC dial is read and simultaneously the temperature of the rotor is found from a thermometer held in thermal contact with the rotor by means of a metal container, filled with water, which fits snugly into a cell hole. Since these measurements are made with the rotor at rest, and since the resistance of the RTIC circuit might change upon rotation of the rotor, this calibration at rest was checked by the diphenyl ether method of Biancheria & Kegeles (1954) in which the rotor revolves at any desired speed.

At first, the diphenyl ether method gave a temperature higher than the other by 1·0±0·1°, at 59 780 rev./min. Later, after replacing the mercury in the lower contact with a fresh, triple-distilled sample, the discrepancy was reduced to 0·2±0·1° at 59 780 rev./min. The same sample of diphenyl ether was used throughout (Dow Chemical Co., Midland, Michigan; perfume grade). However, measurements made during this period of the sedimentation coefficient of bovine albumin show better agreement if the temperature is taken from the calibration at rest than if the results of the diphenyl ether method are used. The reasons for this are not understood at present. Temperatures quoted here are based on the calibration at rest. The sedimentation coefficients were measured in experiments when the discrepancy was 0·2°: the uncertainty in S corresponding to an uncertainty of 0·2° is 0·4%. The diffusion coefficients were measured from earlier experiments when the difference between the results of the two methods was 1·0°; the diffusion coefficients reported here would be decreased by roughly 2% if the diphenyl ether temperatures were used instead. (The precision of measuring diffusion coefficients in the manner described here is, at best, only 5%.)

Speed measurement and instrument constants. In each experiment, the speed of rotation given by the odometer readings at the beginning and end of the period at full speed agreed with the set speed (59 780 rev./min.) within the experimental error of taking the odometer readings (<0·1%). The odometer records the number of revolutions of the rotor times 1/64000; dividing 64000 times the difference in odometer readings by the corresponding time interval gives the average speed.

The distance of the index hole from the centre of rotation increased by 0·035 cm. as the rotor was brought to full speed; this was measured in the manner suggested by Kegeles & Gutter (1951). At rest, the distance from the outer (flat) edge of the inner index hole to the centre of rotation is 5·715 cm.; this was found by measuring the outer diameter of the rotor with a micrometer and then the distance from the rotor edge to the index hole by means of a travelling microscope.

With the use of the ruled disk provided with the instrument, the magnification factor F (for distance in the direction of sedimentation) was found to be 0·4283±0·0002 (cm. in cell/cm. on plate) with the old drive and 0·4289±0·0003 with the new drive. This was measured with the rotor at rest and would not take into account any lens effects produced by distortion of the cell under running conditions.

Measurement of the boundary-gradient curve. A diagonal wire schlieren system was used: cf. Fig. 1. To read the photographic plates, a travelling microscope (Gaertner toolmaker's microscope M2001 RS) with a two-way movement and a projection attachment (M2001 PA) was used. The height of the gradient curve was measured, at a given position, at the centre of the pattern—no attempt was made to take readings at the upper or lower edges. This subject, as well as the use of reference base lines, is discussed in detail in the preceding paper.

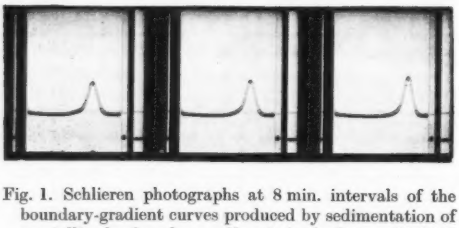

Fig. 1. Schlieren photographs at 8 min. intervals of the boundary-gradient curves produced by sedimentation of crystalline bovine plasma albumin in the Spinco Model E ultracentrifuge. Speed 59 780 rev./min., initial concentration 0·67 g./100 ml., diagonal wire angle 55° (on the basis of zero magnification at 90°). Sedimentation from right to left.

CALCULATIONS

It is generally accepted, in studying free diffusion by means of refractive index-gradient curves, that the ratio of height to area of the boundary curve yields the most precise value for D. Consequently, it is desirable to use this ratio in measuring D from sedimentation-velocity experiments, and Fujita (1956) has derived the necessary equation for doing this. His equation (47) may be written as

$$\left(\frac{AF(\xi_m)}{H}\right)^2 = \frac{2D}{\omega^2 S_0}(e^\tau - 1)\,[1+(1-\lambda)^{\frac{1}{2}}]^2, \qquad (1)$$

where

$$\frac{A}{H} = \int_{x_0}^{x_w} (\partial c/\partial x)\,dx/(\partial c/\partial x)_{\max.}$$

$$= \int_{x_0}^{x_w} (\partial n_c/\partial x)\,dx/(\partial n_c/\partial x)_{\max.}, \qquad (1a)$$

$$\xi_m = \left[\frac{\omega^2 S_0 x_2^0 k c_0}{2D}\right]^{\frac{1}{2}} \left[\frac{\lambda^{\frac{1}{2}}}{1+(1-\lambda)^{\frac{1}{2}}}\right], \qquad (1b)$$

$$F(\xi_m) = \frac{\Phi'(\xi_m)}{1+\Phi(\xi_m)} + 2\xi_m, \qquad (1c)$$

$$\Phi(\xi_m) = \frac{2}{\sqrt{\pi}} \int_0^{\xi_m} e^{-y^2}\,dy, \qquad (1d)$$

$$\lambda = kc_0(1-e^{-\tau}), \qquad (1e)$$

$$\tau = 2\omega^2 S_0 t, \qquad (1f)$$

$$S = S_0(1-kc). \qquad (1g)$$

The symbol $\partial n_c/\partial x$ refers to that portion of the refractive-index gradient caused by the variation in protein concentration with distance (cf. preceding paper). (It is assumed that the specific refractive increment is independent of concentration and pressure.) Distance, x, is measured from the centre of rotation; x_0 is the position of the meniscus and x_w marks a position on the other side of the boundary where $\partial c/\partial x = 0$. Concentration is measured on a weight/volume basis and c_0 is the initial concentration of the sample, before sedimentation has begun. The solute's sedimentation coefficient at infinite dilution is S_0, D is its diffusion coefficient (assumed constant), t represents time and ω is the angular speed of rotation.

Equation (1) may be used to find D simply by plotting, for a given sedimentation experiment, $[AF(\xi_m)/H]^2$ against $(e^\tau - 1)\,[1+(1-\lambda)^{\frac{1}{2}}]^2$, measuring the slope of this plot and then multiplying the slope by $\omega^2 S_0/2$. For brevity, this plot will be referred to as the height–area plot. To obtain the values required for this plot, one must determine A and H from each schlieren photograph and one must know the value of τ at the time of each photograph; λ and ξ_m both are computed from τ and from quantities which are constant during a given sedimentation experiment. In order to evaluate S_0, which enters into τ, and k, which enters into λ and ξ_m, one must perform a series of sedimentation experiments at different concentrations and find the rate of movement of the boundary at each concentration.

Measurement of the area under the gradient curve. Assuming that the photographic plates have been measured and reference base lines subtracted, one begins by computing the areas under the boundary-gradient curves.

$$A = \Delta x\, \Sigma(\partial n_c/\partial x), \qquad (2)$$

where $(\partial n_c/\partial x)$ values may be left in the arbitrary units in which they are measured from the photographic plates since the units cancel in the ratio A/H. The distance between successive $(\partial n_c/\partial x)$ readings is Δx, which is measured in units of cm. in the cell. The error introduced by using trapezoidal integration (equation 2) to find A is readily made less than 0·1% (cf. the preceding paper).

Values of A decrease with time because of the dilution which occurs upon sedimentation in a sector-shaped cell and changing field. In order to compare values of A from successive photographs, it is necessary to correct for this dilution effect. Multiplying A by $(\bar{x}/x_0)^2$, where $\bar{x}$ is the position of the first moment, gives (to a good approximation) the constant A_0 (cf. the preceding paper):

$$A_0 = A(\bar{x}/x_0)^2. \qquad (3)$$

If the boundary-gradient curve is symmetrical, $\bar{x}$ coincides with x_H, the position of the maximum gradient H.

Since Fujita (1956) has shown that for a linear dependence of S on c, the gradient curve for a single component is quite symmetrical, marked asymmetry in itself indicates heterogeneity. With the bovine albumin studied here, it was necessary to make allowance for the presence of a leading component. When values of $(\partial n_c/\partial x)$ on the trailing side of x_H were substracted from those on the leading side, spaced at corresponding distances from x_H, it was found that the trailing edge of the leading component's gradient curve was zero at x_H. Consequently, H could be taken to include only the gradient of the main component and A_0 for the main component was estimated by computing the area on the trailing side of x_H (again by trapezoidal integration), doubling this area and then multiplying it by $(x_H/x_0)^2$. In Table 2 this quantity is denoted by $(A_0)_1$, and A_0 is the corrected area under the entire gradient curve, obtained by equation (3). This method of finding $(A_0)_1$ is valid when Faxén's (1929) equation applies. A study of its validity for concentration-dependent systems must wait for solution of the continuity equation for the case of two concentration-dependent solutes. In the meantime, it should not be used for markedly concentration-dependent systems.

In order to prevent the fluctuation of values found for $(A_0)_1$ from successive photographs from appearing in the height–area plot, and perhaps obscuring any upward curvature, the values for A used in the computations are calculated from the average of the values for $(A_0)_1$ by

$$A = (x_0/x_H)^2(\overline{A_0})_1. \qquad (4)$$

Evaluation of the diffusion coefficient. Once values have been obtained for A, H, ω, S_0, τ, x_0, k and c_0, the calculations are simple and straightforward, except that one must assume a value for D in order to calculate ξ_m. If the measured value of D is significantly different from the assumed value, then one must continue with successive approximations until the two values of D agree. In computing $F(\xi_m)$, *Tables of Probability Functions* (Vol. I, Federal Works Agency, Works Projects Administration, City of New York, 1941) are very useful (available from the Superintendent of Documents, Government Printing Office, Washington, D.C., U.S.A.).

Finally, D is found from the slope of the height–area plot. The point (0, 0) does not necessarily fall on this plot because ω, and thus the solute's sedimenting velocity, is changing as the rotor comes up to speed and because during this period the process of diffusion is restricted rather than free diffusion. These effects compensate to some extent and in both

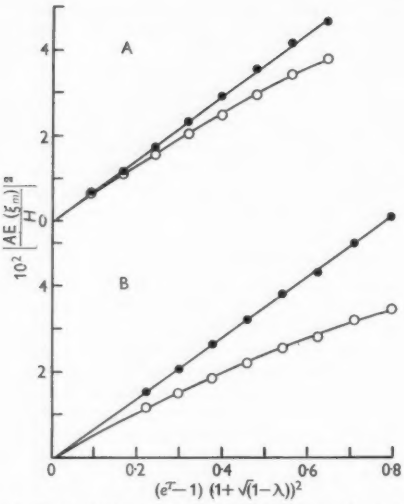

Fig. 2. The height–area plot from which D is evaluated: its slope is $2D/\omega^2 S_0$. Points represented by filled circles have been corrected for concentration dependence; the open circles show the same data without this correction. Fig 2 A (above): $c_0 = 0{\cdot}67$ g./100 ml.; Fig. 2 B: $c_0 = 1{\cdot}35$ g./100 ml.

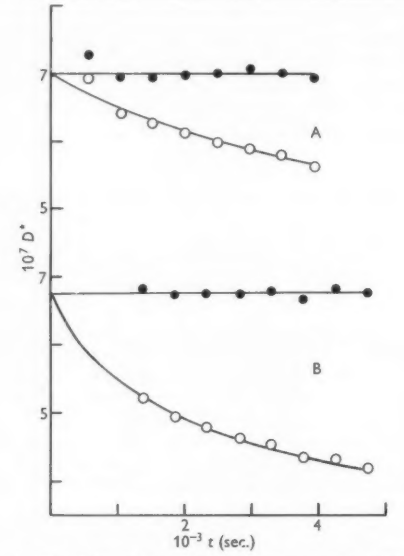

Fig. 3. The data of Fig. 2 plotted as apparent diffusion coefficients. Filled circles again show data corrected for concentration dependence, open circles show the same data without this correction. Fig. 3 A (above): $c_0 = 0{\cdot}67$ g./100 ml.; Fig. 3 B: $c_0 = 1{\cdot}35$ g./100 ml.

Figs. 2 A and B it may be seen that the best straight line through the various points passes close to the point (0, 0). The plots of the apparent diffusion coefficient, D^*, against t in Fig. 3 are illustrative only and were not used in finding D. [The values of D^* in Fig. 3 are obtained by dividing the ordinates of Fig. 2 by the corresponding abscissae times $(2/\omega^2 S_0)$, after subtracting from the abscissa the intercept of the plot.]

Computations when the solute is heterogeneous. When the Oxford experiments were computed according to equation (1), upward curvature was observed in each of the height–area plots, indicating that in this sample of albumin the main component itself is heterogeneous. Since in this case the intercept of the height–area plot cannot be found by placing the best straight line through the data, it was assumed that the curves pass through the point (0, 0) and the D^* values of Fig. 4 were computed on this basis.

To examine the question of whether the curvature is significant, and whether the appearance of heterogeneity (Fig. 4) could be caused instead by the requirement that the height–area plot pass through the point (0, 0), one may proceed in the following way. First equation (1) is re-

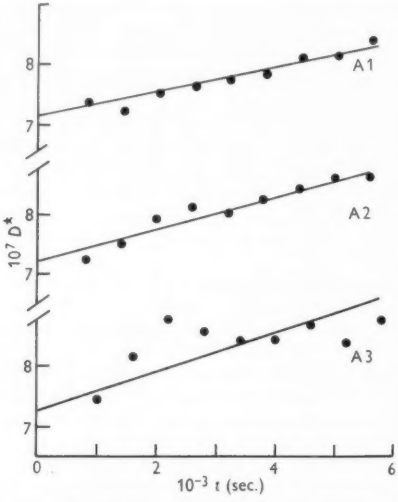

Fig. 4. Some older data for another sample of albumin, recalculated for this paper as apparent diffusion coefficients (corrected for the dependence of S on c) against time. In each case, the intercept is the value of D predicted from the diffusion measurements of Gosting (1955). All three experiments were carried out with the same solution ($c_0 = 0{\cdot}61$ g./100 ml.) in order to check their reproducibility.

written, after expanding $(e^{\tau}-1)$ into a Maclaurin series, as

$$W = 2Dt + 2DS_0\omega^2t^2 + \tfrac{4}{3}DS_0^2\omega^4t^3 + \ldots, \quad (5)$$

where $$W \equiv \frac{1}{2}\left\{\frac{AF(\xi_m)}{H[1+(1-\lambda)^{\frac{1}{2}}]}\right\}^2. \quad (5a)$$

Let W at the time of the first photograph (t_0) be denoted by W_0:

$$W_0 = 2Dt_0 + 2DS_0\omega^2t_0^2 + \tfrac{4}{3}DS_0^2\omega^4t_0^3 + \ldots. \quad (5b)$$

Next subtract equation (5*b*) from (5) and sum over all values of W (one for each schlieren photograph, or $n+1$):

$$\sum_{i=0}^{n}(W_i - W_0) = 2D\sum_{i=0}^{n}(t_i - t_0) + 2DS_0\omega^2\sum_{i=0}^{n}(t_i^2 - t_0^2) + \tfrac{4}{3}DS_0^2\omega^4\sum_{i=0}^{n}(t_i^3 - t_0^3) + \ldots. \quad (6)$$

If there is heterogeneity, the right-hand side of equation (6) should be significantly less than the left-hand side. For the non-concentration-dependent case when the distribution of S is Gaussian (cf. Baldwin, 1954), the difference would be approximately

$$p^2\omega^4x_0^2\left\{\sum_{i=0}^{n}(t_i^2 - t_0^2) + S_0\omega^2\sum_{i=0}^{n}(t_i^3 - t_0^3)\right\},$$

where p is the standard deviation of S_0. To use equation (6), one must know D from independent diffusion measurements and one must also know t_0 to find the terms in $(t^2 - t_0^2)$ and $(t^3 - t_0^3)$. However, these are minor terms and a small error in t_0 is unimportant; for the major term, involving $(t - t_0)$, the value of t_0 itself is not required.

Measurement of sedimentation coefficients. It has been customary to measure sedimentation coefficients from the slope of the plot of $\ln(x_H/x_0)$ against ω^2t and to refer to the sedimentation coefficient measured in this way as being that at the initial concentration of the experiment. In recent years, two features of this method have been examined critically. Goldberg (1953) showed that the true boundary position, x_b, is given by a moment equation and that x_b differs slightly from x_H for a symmetrical gradient curve. Kegeles & Gutter (1951) pointed out that, since the concentration changes during the experiment, it would be more logical to refer to the sedimentation coefficient measured in this way as being that at the concentration found midway through the experiment. Alberty (1954) noted that one effect of the dependence of S upon c is to introduce curvature into the plot of $\ln(x_b/x_0)$ against ω^2t; he suggested that this should be taken into account in computing S and gave equations for doing this. From a solution of the continuity equation, Fujita (1956) obtained an equation relating $\ln(x_H/x_0)$ to ω^2t for a linear dependence of S on c and Trautman, Schumaker, Harrington & Schachman (1954) have given a method for computing S which takes into account both concentration dependence and variation in temperature during an experiment.

In view of the accurate speed and temperature control now available, it seemed worth while to study methods of computing S to determine whether full use is being made of the experimental accuracy. When S is a quadratic function of c

$$S = S_0(1 - k_1c - k_2c^2), \quad (7)$$

the equation relating $\ln(x_b/x_0)$ to ω^2t for a homogeneous solute is (cf. Alberty, 1954, and preceding paper)

$$\ln(x_b/x_0) - (S^\dagger)(S_0)(k_1c_0 + 2k_2c_0^2) + \ldots = S^\dagger\omega^2t, \quad (8)$$

where $S^\dagger$ is the sedimentation coefficient at the beginning of the experiment

$$S^\dagger \equiv S_0(1 - k_1c_0 - k_2c_0^2). \quad (8a)$$

If one defines an apparent sedimentation coefficient, S^*, by the equation

$$S^* \equiv \{\ln(x_b/x_0)_t - \ln(x_b/x_0)_{t_0}\}/\omega^2(t - t_0), \quad (9)$$

where $(t - t_0)$ is the time from the first photograph, then S^* is easily calculated (cf. Table 1) as well as being based on a time origin which can be measured precisely.

Table 1. *Sample calculation of S^**

$10^2\log(x_H/x_0)$	$10^2Dt/2{\cdot}303x_0^2$	$10^2\log[(x_b)_t/(x_b)_{t_0}]^a$	$10^{-3}(t-t_0)^{b,d}$	$S^*_{25,w}$ (s)^c
0·557	0·001	0	0	—
2·473	0·003	1·918	2·40	$4{\cdot}79_5$
2·853	0·003	2·298	2·88	$4{\cdot}78_9$
3·242	0·003	2·687	3·36	$4{\cdot}79_9$
3·627	0·004	3·073	3·84	$4{\cdot}80_1$
4·005	0·004	3·451	4·32	$4{\cdot}79_3$

[a] $\log[(x_b)_t/(x_b)_{t_0}] = \log(x_b/x_0)_t - \log(x_b/x_0)_{t_0}$, where x_b is the boundary position, x_0 is the position of the meniscus and t_0 is the time of the first photograph. According to Faxén's (1929) equation, $\ln(x_b/x_0) = \ln(x_H/x_0) + Dt/x_0^2 + \ldots$.

[b] The time from the first photograph.

[c] $S^* \equiv 2{\cdot}303\log[(x_b)_t/(x_b)_{t_0}]/\omega^2(t-t_0)$. $S^*_{25,w} = S^*\left(\frac{\eta}{\eta_{25,w}}\right)\frac{(1-\bar{v}\rho)_{25,w}}{(1-\bar{v}\rho)}$.

[d] In order to plot $S^*_{25,w}$ against $t + t_0$, it is necessary to find t_0 and to add $2t_0$ to $t - t_0$. For this purpose, t_0 may be estimated with satisfactory accuracy as $\log(x_b/x_0)_{t_0}$ multiplied by the initial value of $(t-t_0)/\log[(x_b)_t/(x_b)_{t_0}]$. Thus for this experiment $t_0 = (0{\cdot}558 \times 10^{-2})(2{\cdot}40 \times 10^3)/(1{\cdot}918 \times 10^{-2})$, or $t_0 = 0{\cdot}70 \times 10^3$ sec.

Comparison of equations (8) and (9) shows that S^* is well-defined theoretically

$$S^* = S^\dagger\{1 + (S_0\omega^2)(t+t_0)(k_1c_0 + 2k_2c_0^2) + \ldots\}, \quad (10)$$

but that it is a function not only of c_0 but also of $(t+t_0)$. One might avoid this difficulty by plotting S^* against $t+t_0$ and extrapolating to $t+t_0=0$ in order to find $S^\dagger$, a function only of c_0 (cf. Alberty, 1954); however, there are experimental difficulties in doing this. Fig. 5 shows the effect on S^* of a fixed uncertainty in reading the positions x_0 and x_H: since the percentage uncertainty in $(x_H - x_0)$ decreases as the distance between x_H and x_0 increases, the values of S^* become increasingly accurate as time increases. Thus it is not desirable to try to extrapolate S^* back to $t+t_0=0$.

By taking values of S^* from a set of experiments at fixed $(S_0\omega^2)(t+t_0)$, these values of S^* are a function only of c_0:

$$S^* = S_0\{1 - k_1c_0[1 - (S_0\omega^2)(t+t_0)] - c_0^2[k_2 - 2k_2(S_0\omega^2)(t+t_0) + k_1^2(S_0\omega^2)(t+t_0)] - \ldots\}. \quad (11)$$

As equation (11) shows, once $(S_0\omega^2)(t+t_0)$ is fixed the plot of S^* against c_0 may be extrapolated to $c_0=0$ in order to find S_0, and then the plot of c_0 against $(S_0 - S^*)/S_0c_0$ yields, from its slope and intercept, quantities from which k_1 and k_2 may be found. When k_1 and k_2 are known, the slope of the plot of S^* against $t+t_0$ is known from equation (10), and the graphs from which the values of S^* were taken can be re-examined, to see whether the lines correspond with theory.

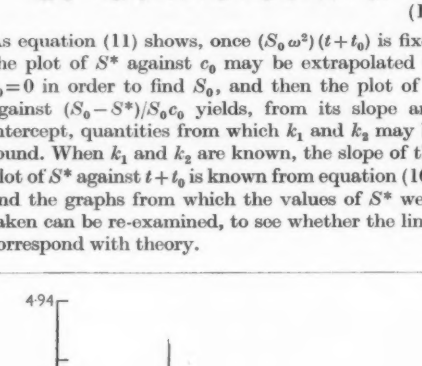

Fig. 5. The plot used to measure S. An 'apparent' sedimentation coefficient, S^*, defined by equation (9), is plotted against $t+t_0$, where t_0 is the time of the first photograph. The line drawn through the points has the theoretical slope given by equation (10). The line through each point gives the uncertainty in S^* caused by an uncertainty of 0·001 cm. in reading the positions of x_H and x_0 on the photographic plate. For this experiment $c_0 = 0{\cdot}87$ g./100 ml.

RESULTS AND DISCUSSION

Values found for the diffusion coefficient

Fig. 2 shows the height–area data from experiments at $c_0 = 0{\cdot}67$ and 1·35 g./100 ml. The slope of Fig. 2A gives a value for D of $7{\cdot}0_2 \times 10^{-7}$, as compared with $6{\cdot}85 \times 10^{-7}$ predicted from the diffusion measurements of Gosting (1955). In Fig. 2B the observed value is $6{\cdot}7_6 \times 10^{-7}$ and the predicted value is $6{\cdot}94 \times 10^{-7}$. It should be remembered that the precision of the values shown in Figs. 2 and 3 is considerably greater than their accuracy. The average value of $(A_0)_1$, from which the values of A were computed, is uncertain by $\pm 2\%$ and this uncertainty is doubled in the data of Figs. 2 and 3 since A is squared in computing D. Thus the observed value of D cannot be expected to be more accurate than $\pm 5\%$, and the agreement between observed and predicted values is satisfactory.

To show more clearly the magnitude of the concentration dependence correction, the data have been replotted in Fig. 3 as apparent diffusion coefficients, D^* (cf. Calculations). By the end of the experiment at $c_0 = 1{\cdot}35$, the uncorrected value of D^* falls almost to half the corrected value. Since 1·35 g./100 ml. is in the range where sedimentation studies of globular proteins are commonly made, and since bovine albumin shows less marked concentration dependence than many, this correction of the apparent diffusion coefficients for the dependence of S on c is clearly an important correction. Values of D^* which have not been corrected for the dependence of S on c cannot be expected to agree with measurements of D from conventional diffusion experiments. Where such agreement has been found (cf. Svedberg & Pedersen, 1940, p. 286, Lauffer, 1942; Davisson, Gibson, Ray & Vestling, 1953) it must now be presumed that the proteins studied were heterogeneous and that this compensated for neglect of the concentration-dependence correction. Dieu & Oth (1954) have reported values of uncorrected apparent diffusion coefficients for bovine albumin which are less than the predicted value of D, but values for ovalbumin which they say are in agreement with the predicted value of D.

It should be emphasized that the presence of a faster sedimenting component in this sample of albumin introduces some uncertainty into the calculation of D by Fujita's equation. The procedure for finding $(A_0)_1$ is based on Faxén's equation, not on an equation which takes into account the dependence of S on c. In other words, the effect of the leading component on the shape of the gradient curve of the main component is not known. Also the procedure for finding k in $S = S_0(1 - kc)$ is based on the assumption that only one component is present. The uncertainty in the calculated value of D increases with increasing concentration, for both

these reasons. Thus the agreement of predicted and observed values for D is more significant in the experiment at $c_0 = 0{\cdot}67$ than at $c_0 = 1{\cdot}35$ g./100 ml. Also, the plot of S against c shows slight downward curvature above a concentration of 1 g./100 ml. However, the precision of measuring the gradient curves is better at the higher concentration.

The predicted value of D was computed in the following way. According to the measurements of Gosting (1955), $\bar{D}$ (the weight-refractive average referred to 25° and the viscosity of water) is $6{\cdot}73_2 \times 10^{-7}$ for this sample at a mean concentration of 0·515 g./100 ml. (Values of D refer to the mean concentration of the solutions on the two sides of the boundary; in the experiments reported here the mean initial concentrations are 0·33 and 0·68 g./100 ml. Creeth (1952) reports that the concentration dependence of D for bovine albumin (at pH 6·8) is negligible in this range of concentration.) Taking α_2 (the fraction, on a refractive increment basis, of the leading component) as 0·12, one may compute from the fringe deviation graph of the diffusion experiment that D_1/D_2 (the ratio of diffusion coefficients of the main and leading components) is 1·40 (L. J. Gosting, private communication). Since $\bar{D} = \alpha_1 D_1 + \alpha_2 D_2$, it follows that $D_1 = 1{\cdot}035\,\bar{D}$ or $6{\cdot}97 \times 10^{-7}$. Values of D for the particular temperature and buffer of the sedimentation experiment were then calculated on the assumption that $D\eta/T$ was constant, where η is the solvent viscosity and T the absolute temperature. The values used for k were 0·057 at $c_0 = 0{\cdot}67$ g./100 ml. and 0·059 at $c_0 = 1{\cdot}35$ g./100 ml.

If the expression for D is taken to be

$$\boldsymbol{R}T(1 + \partial \ln y/\partial \ln c)/f$$

(cf. Onsager & Fuoss, 1932), where f is the frictional coefficient and y is the activity coefficient on the c scale, then a slight pressure dependence of D is to be expected. The viscosity of the solvent, which affects f, depends on pressure and the thermodynamic term also is, in general, a function of pressure (cf. Guggenheim, 1950).

Evidence of heterogeneity in one sample

Fig. 4 shows three experiments carried out with a single solution of bovine albumin. A different batch of albumin (Armour, lot no. 10 522) was used from the one considered above, the pH was lower (4·34), and the measurements were made with the Oxford oil-turbine ultracentrifuge. The fractional amount of the leading component, α_2, was found to be 0·11 by the procedure outlined in Calculations. In Fig. 4 it is apparent that the values of D^* increase with time: this indicates heterogeneity (cf. Williams, Baldwin, Saunders & Squire, 1952; Baldwin, 1954). However, the solution to the differential equation has not been carried out for a heterogeneous concentration-dependent system; so there is no theoretical expression for the slope of Fig. 4. For a solute without dependence of S on c and a Gaussian distribution of S, the slope would be approximately $(\frac{1}{2})p^2\omega^4 x_0^2(1 + \bar{S}_0\omega^2 t + \ldots)$ (Baldwin, 1954; equation (5) which, combined with equation (1), gives $\partial(C/C_0)/\partial x$ when g(S) is Gaussian, contains a misprint: the term $p^* 2\sqrt{\pi}$ should be $p^* \sqrt{2\pi}$). Taking this as the expression for the slope gives a value for $p_{25,\,w}$ of 0·35 s ± 0·04 s (S.D.), or 7% of S_0 (s = Svedberg units). The alternative calculation of p from the difference between the left-hand and right-hand sides of equation (6) gives

$$p_{25,\,w} = 0{\cdot}36\text{ s} \pm 0{\cdot}04\text{ s (S.D.)}.$$

This agreement between the two values of p rules out the possibility that the upward slope of D^* against t in Fig. 4 could have been caused by requiring the height–area plots to pass through the point (0, 0). Since a difference of 5% between D^* and the predicted value of D would not be considered significant, the value for p given above is near the limit of detectability.

The drift with time of D^* would be evident in Expts. A 1 and A 2 even if D were not known from independent evidence. However, in Expt. A 3 the scatter is such that this drift might be overlooked; if one were to calculate D from this experiment as if there were no heterogeneity, the value would be 10% too high. This emphasizes the need for accurate data in using sedimentation measurements to find D, and it also illustrates the difficulty of finding D from sedimentation experiments when the solute is heterogeneous. Even in Expts. A 1 and A 2, where the heterogeneity is evident, the uncertainty in the intercept is considerably greater than it would be with comparably precise data for a homogeneous solute.

Area measurements

Table 2 shows the results of measuring areas under the boundary-gradient curves. The agreement of the values found from successive photographs, after correction for the dilution effect, is quite good in view of the theoretical and practical uncertainties of the schlieren optical system (cf. Kegeles & Gosting, 1947; Trautman & Burns, 1954) and the difficulties of establishing the correct base lines. As Table 2 shows, there is no significant dependence on time of the areas once a correction for the dilution effect has been made. The ratio, α_1, of $(A_0)_1$ to A_0 (cf. Table 2) was found to be $0{\cdot}89_4$ at $c_0 = 0{\cdot}67$ and $0{\cdot}88_5$ at $c_0 = 1{\cdot}35$ g./100 ml. If a correction for the Johnston–Ogston effect (Johnston & Ogston, 1946) is made by their (approximate) equations (2) and (3), these values for α_1 become $0{\cdot}88_8$ at $c_0 = 0{\cdot}67$ and $0{\cdot}87_4$ at $c_0 = 1{\cdot}35$ g./100 ml.

Table 2. *Areas from schlieren diagrams*

Measurements from two experiments of the area under the boundary-gradient curve times the correction for the dilution effect. The areas were found by trapezoidal integration; values given here are in arbitrary units. The left-hand set of columns gives the area under the entire curve, A_0, and the right-hand set shows that attributed to the main component, $(A_0)_1$. The diagonal-wire angle gave a higher magnification (by a factor of $1{\cdot}50_2$) at the lower concentration; the areas from the experiment at $c_0 = 0{\cdot}67$ agree, within the precision of the measurements, with those from the experiment at $c_0 = 1{\cdot}35$ g./100 ml.

Photograph no.	(*a*) A_0	(*b*) A_0	(*a*) $(A_0)_1$	(*b*) $(A_0)_1$
1	95·7	127·3	85·5	115·0
2	94·6	125·9	85·1	111·0
3	94·9	127·2	84·1	111·6
4	92·7	127·7	83·3	112·4
5	93·4	126·4	82·5	111·2
6	93·5	126·3	84·4	111·7
Average	94·1±1·2%	126·8±0·6%	84·1±1·3%	112·2±1·4%
	(*a*) $c_0 = 0{\cdot}67$ g./100 ml.		(*b*) $c_0 = 1{\cdot}35$ g./100 ml.	

Table 3. *Sedimentation coefficients of bovine albumin*

c_0[a]	$S_{25,w}$[b]	$S_{calc.}$[c]
0·29	$4{\cdot}92_7$	4·930
0·59	$4{\cdot}83_9$	4·843
0·87	$4{\cdot}77_2$	4·759
1·17	$4{\cdot}66_3$	4·667
1·17	$4{\cdot}66_2$	4·667
1·76	$4{\cdot}47_5$	4·472

[a] The initial concentration of the experiment in g./100 ml.

[b] The measured sedimentation coefficient at the initial concentration of the experiment, in Svedberg units (s).

[c] Calculated from the equation $S_{25,w} = 5{\cdot}01\ (1 - 0{\cdot}054c - 0{\cdot}004c^2)$ s.

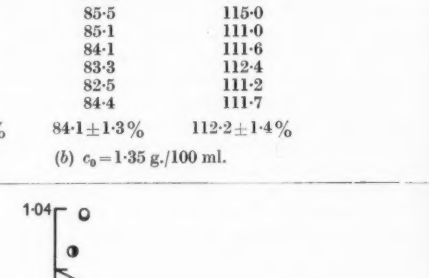

Fig. 6. Comparison of the results for the concentration dependence of S with some of the data in the literature. Open circles, data of Kegeles & Gutter (1951); half-filled circles, data of Koenig & Pedersen (1950); filled circles, this paper. To compare measurements of the concentration dependence of S rather than of absolute values, all sedimentation coefficients were divided through by S_0, the sedimentation coefficient for infinite dilution. The values taken from the literature are plotted against the average concentration of the experiment. (For the data of Koenig & Pedersen, the average concentration was taken to be 0·89 time the initial concentration.)

Precision of the sedimentation coefficients

Several studies have recently been made of the concentration dependence of S for bovine albumin (Koenig & Pedersen, 1950; Kegeles & Gutter, 1951; Creeth, 1952; Miller & Golder, 1952; Taylor, 1952; Shulman, 1953). Yet, on examination of the data in these papers, it became apparent that in none of them, not even in the very careful study of Kegeles & Gutter, was the slope of S against c known with the accuracy required for these boundary-spreading calculations. Consequently, a study of the concentration dependence of S was made; the results are given in Table 3. In Fig. 6, the need for such a study may be seen; values given by various authors have been plotted as S/S_0 against c. This plot was chosen, instead of S against c, in order to focus attention on the accuracy with which the concentration dependence is known rather than on the absolute values of S.

The sedimentation coefficients shown in Table 3 appear to be considerably more precise than any yet reported for albumin. As the third column of the table shows, they fit the equation

$$S_{25,w} = 5{\cdot}01(1 - 0{\cdot}054c - 0{\cdot}004c^2)\ \text{s},$$

with a maximum deviation of $0{\cdot}2_6$% and a standard deviation of $0{\cdot}1_4$% of S_0. In analysing reasons for the precision of these values, three factors should be mentioned. First, the control of temperature by the RTIC unit (±0·02°) is better than in the earlier studies on albumin. Secondly, the precision gained by the use of a travelling microscope, as compared to the use of enlarged tracings, is considerable, particularly when the microscope has a two-way movement so that the plates may be aligned accurately. Thirdly, the method of calculation makes good use of the experimental accuracy.

There appears to be downward curvature in the plot of S against c, so that plotting $1/S$ against c would make the curvature greater, not less. Cecil & Ogston (1949), in their very accurate study of the sedimentation behaviour of β-lactoglobulin, observed this same kind of curvature so that one may wonder if it is not a general property of the globular proteins. If curvature is overlooked and the extrapolation for finding S_0 based on data from too high a concentration range, the value of S_0 will be too high.

Values of S^* were read from the graphs of S^* against $t+t_0$ at $t+t_0 = 5{\cdot}76 \times 10^3$ sec. After S_0 had been found from the plot of S^* against c_0, values of $S^\dagger$ (the sedimentation coefficient at the initial concentration c_0) were calculated from equation (10). The values of S were converted to the viscosity and density of water at 25°, rather than at 20°, because Longsworth (1954) has shown a slight dependence of f/η (where f is the frictional coefficient) on temperature.

Molecular weight of bovine albumin

Use of the Svedberg equation (Svedberg, 1925) gives a molecular weight of 66 500 with the values at pH 4·6 of

$$S_{25,\,w} = 5{\cdot}01 \times 10^{-13} \text{ sec.},$$
$$D_{25,\,w} = 6{\cdot}97 \times 10^{-7} \text{cm.}^2 \text{ sec.}^{-1}$$

[the value of Gosting (1955), corrected for the presence of a slower-diffusing impurity by means of the fringe deviation graph of the diffusion experiment and by sedimentation analysis for the amount of the impurity], $\bar{v} = 0{\cdot}734$ ml. g.$^{-1}$ (Dayhoff *et al.* 1952) and ρ for $H_2O = 0{\cdot}9971$ at 25°. It should be pointed out that no attempt has been made to correct the values of S for pressure dependence and that this correction may well be significant. For a comparison of these values for M, S, and D with other values in the literature, see Harrington, Johnson & Ottewill (1956).

SUMMARY

1. The use of Fujita's theory for the concentration-dependent sedimentation of a single solute is discussed and illustrated with experiments on bovine plasma albumin.

2. With this theory, the diffusion coefficient of bovine albumin has been measured from experiments with the velocity ultracentrifuge. The values obtained for D were in agreement within experimental error ($\pm 5\%$) with the value known from Gosting's diffusion studies.

3. As the theory shows, the correction of apparent diffusion coefficients for the dependence of S on c is large. Previous work, in which this correction was ignored, should be re-evaluated.

4. The main component of one sample of bovine albumin showed measurable heterogeneity in sedimentation behaviour.

5. A method for calculating sedimentation coefficients is discussed. The method takes into account the dependence of S on c and the effect on S of the uncertainty in measuring the photographic plates.

6. Sedimentation coefficients of bovine albumin measured from six experiments fit the equation $S_{25,\,w} = 5{\cdot}01\,(1 - 0{\cdot}054c - 0{\cdot}004c^2)$ s with a standard deviation of $0{\cdot}1_4\%$.

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A Chromatographic Method of Analysis for Thiols

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Although there are a number of methods for the detection and estimation of thiol compounds (Patterson & Lazarow, 1954), no fully adequate chromatographic procedure has been hitherto available. The reduced forms of coenzyme A (Stadtman, 1952), cysteine (Toennies & Kolb, 1951), and glutathione (Miller & Rockland, 1952) have been detected on paper by means of thiol reactions, but the chief obstacle has been the ease of autoxidation of the thiol groups, leading to secondary spots and the excessive formation of 'comets' or tailing.

The specific problem of α-amino acid–thiol separations was first resolved by Hanes, Hird & Isherwood (1950) and expanded by Gutcho & Laufer (1954). In their methods the thiol group is masked with *N*-ethylmaleimide (Friedman, Marrian & Simon-Reuss, 1949), a reaction that proceeds rapidly to completion in dilute aqueous solution over a wide pH range (Leopold & Price, in preparation). After chromatographic development of the *N*-ethylmaleimide–thiol conjugates, the compounds are detected with ninhydrin. A disadvantage of this method was that these conjugates were distinguished from other amino acids and peptides only by their R_F values. More recently (Benesch, Benesch, Gutcho & Laufer, 1956) a method has been found for detecting the conjugates of *N*-ethylmaleimide and any thiol. An alternative approach, suggested by Gutcho & Laufer (1954), is that the thiol-masking reagent contains a potential chromophore. Such a procedure is in fact the basis of a histochemical technique which has been developed successfully (Barrnett & Seligman, 1954). The most promising substance of this sort, described recently

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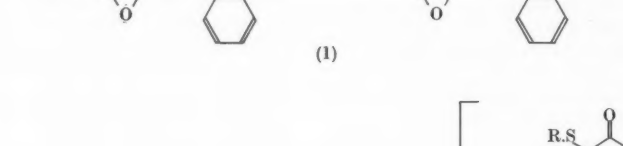
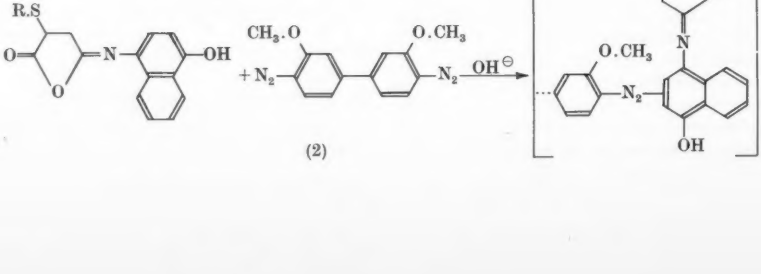


(1)



(2)

by Tsou, Barrnett & Seligman (1955), is *N*-(4-hydroxy-1-naphthyl)*iso*maleimide. The *iso*maleimide moiety reacts rapidly and specifically with thiols (equation 1), whereupon the α-naphthol moiety may be combined with diazotized reagents to yield intensely coloured dyes (equation 2).

The method described below consists in the preparation of the *N*-(4-hydroxy-1-naphthyl)*iso*maleimide derivatives of thiols followed by chromatography and detection with tetraazotized di-*o*-anisidine.

A preliminary report of this work has appeared elsewhere (Price, 1956).

The following abbreviations are employed in this paper: cysteine, CySH; glutathione, GSH; naphthalene-1-acetic acid, NAA; oxidized glutathione, GSSG; *N*-(4-hydroxy-1-naphthyl)*iso*maleimide, HNI; tetraazotized di-*o*-anisidine, TDA.

METHODS

Materials

'Naphthanil diazo blue B' (Dupont), obtained from Fisher Scientific Company, St Louis, Mo., U.S.A., was used as a source of tetraazotized di-*o*-anisidine (TDA).

Cysteine (CySH), glutathione (GSH) and oxidized glutathione were products of Schwarz Laboratories; commercial preparations of homocysteine, djenkolic acid, *n*-butylthiol and thiomalic acid were gifts of Dr R. Henze. The sample of cysteinylglycine was a gift from Dr L. Laufer of the Schwarz Laboratories, Mount Vernon, N.Y., U.S.A. Thioctic acid (6:8-dimercaptooctanoic acid) was a gift of Dr L. Reed. Thiocoumarin was a gift from Dr R. Goodwin. Coenzyme A (approx. 75%) was a gift from the Pabst Laboratories, Milwaukee, Wis., U.S.A. It was reduced with potassium borohydride immediately before use (Jones, Black, Flynn & Lipmann, 1953).

Chromatographic solvents were all of the highest commercial grades available, but were not specially purified.

Krylon, a methacrylate solution, is manufactured by Krylon, Inc., Philadelphia 46, Pa., U.S.A.

The sample of *N*-(4-hydroxy-1-naphthyl)*iso*maleimide (HNI) principally used was a generous gift from Dr A. Seligman and was part of the original batch described by Tsou *et al.* (1955). Dr L. Laufer, of the Schwarz Laboratories, also kindly supplied two samples and one lot was prepared in this laboratory.

Reactions and purification of HNI. The original sample of HNI dissolved in ethanol and chromatographed in butanol–water contained at least two components (Fig. 1). When increasing amounts of thiol were made to react with the reagent (for conditions, see below) and the products chromatographed, the more rapidly moving component was observed to disappear in proportion to the appearance of a new spot. The slower-moving component did not change in intensity (this is not evident from the tracing) and was concluded, therefore, to be a contaminant.

Except in the solvent systems rich in butanol, the presence of the contaminant was not a serious disadvantage, since both contaminant and reagent moved close to the solvent front. Where the contaminant might have obscured other components, a purified HNI reagent was employed. This was prepared by streaking a large quantity of HNI on a sheet of Whatman no. 20 filter paper, chromatographing in *n*-butanol–water and eluting the faster component with ethanol. The material obtained no longer gave the 'base reaction' (see below), but was free of contaminant and still reacted with thiols (Fig. 1).

In several preparations of HNI, the reagent contained widely varying quantities of contaminant. Further work is therefore required to standardize the chemical preparation. Moreover, to the extent that the structural formula (Tsou *et al.* 1955) was deduced from the properties of an impure sample, some reservations must attach to the assigned structure.

One property of HNI observed by Tsou *et al.* (1955) was the formation in aqueous alkaline solution of an intense transient red colour. This 'base reaction' is irreversible and corresponds to the loss of an absorption maximum at 430 mμ. In our experiments upon prolonged exposure to water at acid or neutral pH there was also a loss in the potential for the 'base reaction' and the 430 mμ. absorption, but altered HNI none the less reacted with thiols as before.

The combination between HNI and thiols was greatly accelerated at increasing pH. With GSH, for example, at pH 8 the reaction was complete within a few seconds; at pH 2, on the other hand, the reaction was only half-completed in 45 min. This dependence of the reaction rate on high pH is in contrast to that of *N*-ethylmaleimide: with the latter reagent the rates are high at all pH values tested, but are accelerated under the more acid conditions (Leopold & Price, in preparation).

The detection reaction between HNI and TDA also proceeds rapidly only under alkaline conditions. Since

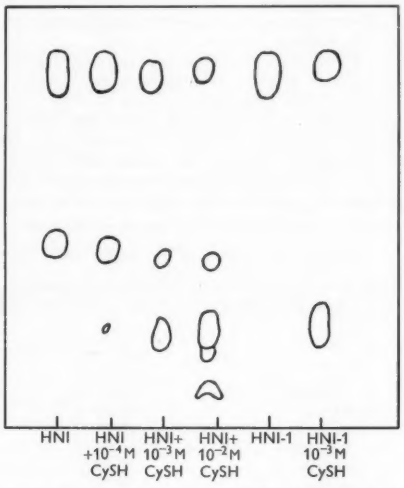


Fig. 1. Reaction of cysteine and HNI. Samples (5 μl.) of 0·1% (w/v) HNI with several concentrations of cysteine (also 5 μl.) chromatographed in solvent 7, ascending. Shown at right: the product of purified HNI (HNI-1) with cysteine is identical with that of unpurified reagent.

hydroxide salts of diazo compounds are known to be readily decomposed in water, it is essential to store the TDA in a weakly acidic solution.

Reagents and solutions required. 'Stock HNI': 0·2% (w/v) of HNI in 95% (v/v) ethanol; it is stable indefinitely at −10°.

'Standard HNI': 0·02% (w/v) of HNI in 50% (v/v) ethanol; stable for at least 1 month at −10°.

TDA: 1 g. of 'naphthanil diazo blue B' was stirred for several minutes with 100 ml. of 0·01 N-HCl in absolute methanol, gravity-filtered through Whatman no. 4 paper, and the filtrate diluted to 1 l. with acetone. The small precipitate that formed overnight was discarded. The initially pale-orange solution slowly decomposed and turned red at −10°, more rapidly at room temperature.

M-$NaHCO_3$ and 2 N-HCl.

Solvent systems for chromatography: solvent 1, 95% (w/v) ethanol–formamide–N acetic acid (75:10:15, by vol.); solvent 2, propan-2-ol–N acetic acid (80:20, v/v); solvent 3, propan-2-ol–N acetic acid (85:15, v/v); solvent 4, propan-2-ol–*n*-butanol–N acetic acid (75:10:15, by vol.); solvent 5, propan-2-ol–*n*-butanol–N acetic acid (45:40:15, by vol.); solvent 6, propan-2-ol–*n*-butanol–formamide (75:10:15, by vol.); solvent 7, water-saturated *n*-butanol.

Procedure

Preparation of marker solutions. To portions (1 ml.) of 'stock HNI' were added 0·6 ml. of water, 0·2 ml. of 10^{-2} M thiol in water and 0·1 ml. of M-$NaHCO_3$. The solution was stirred and at the end of 1 min. the 'base reaction' was stopped with 0·1 ml. of 2 N-HCl. (If an excess of thiol were present, the red colour of the 'base reaction' product bleached within 1 sec. This was avoided as it led to multiple spot formation; cf. Fig. 1.) Marker solutions thus prepared were mM with respect to thiol and about 3 mM in excess of HNI. When stored at −10°, no deterioration was observed over many weeks.

Extraction of free thiols from plant tissues as conjugates with HNI. Samples of about 200 mg. fresh wt. of tissue were placed in conical glass homogenizers of 3 ml. working capacity; 1 ml. of 'standard HNI' was added, followed by 0·1 ml. of M-$NaHCO_3$. Grinding was completed in 1 min. and the reaction stopped with 0·1 ml. of 2 N-HCl. The contents of the homogenizer were transferred to a 5 ml. calibrated and ground-glass-stoppered centrifuge tube with the aid of sufficient 50% (v/v) ethanol to bring the volume to 2 ml. If further treatment were necessary to coagulate proteins, the tubes were placed in a boiling-water bath for 5 min. before final adjustment of volume. The tubes were then centrifuged at 1500 *g* for 10–20 min. and the supernatants collected.

Chromatography. Whatman filter paper no. 20 was used almost exclusively for chromatography, although no. 5 was perhaps equally satisfactory. With faster-running grades the spots were not sufficiently compact. In general, circumstances that led to a rate of movement of the solvent exceeding 20 cm./24 hr. produced elongate spots.

On a sheet of paper (18½ in. × 22½ in.) a series of line segments were drawn 1 cm. long, 2 cm. apart and 12 cm. from the long edge of the paper. For routine screening of solvents, 5 μm-moles (5 μl. of mM marker solutions) gave spots of convenient size. The samples were applied along the line segments either with an Agla micrometer syringe (Burroughs Wellcome Inc., Tuckahoe, N.Y., U.S.A.) or with calibrated capillary micropipettes, prepared according to the directions of Mr R. Hems (personal communication). The paper was dried with a stream of cool or warm, but not hot, air from a hair dryer. (Decomposition of the HNI–thiol conjugates occurred at elevated temperatures.)

With plant-tissue extracts (10%, w/v) about 20 μl. was usually found to provide a detectable spot corresponding to glutathione.

The chromatograms were allowed to develop in the usual manner for 24–48 hr., by which time the front had descended 20–40 cm. beyond the starting lines. Pre-equilibration of the paper with solvent vapours was not found to be advantageous. The chromatogram was then dried in air. To detect the thiol–HNI conjugates, the papers were exposed briefly to NH_3 vapour, dipped into the TDA solution, and hung in a vertical position with the starting line upwards. Within a few seconds blue or red spots appeared.

The pigments formed were stable for months, but spraying with a preservative (Krylon) was sometimes employed.

RESULTS

Results with marker solutions. Cysteine and glutathione, the most common naturally occurring thiols, were readily separated with a number of solvent systems containing ethanol or propan-2-ol

Table 1. *R_F values of* N-(4-*hydroxy*-1-*naphthyl*)iso*maleimide* (*HNI*) *derivatives of various thiols*

All samples were applied on Whatman no. 20 paper in 5 μm-mole quantities; except for solvent 6, descending development was used throughout. For details of solvent systems see Methods.

	R_F value in				
Component	Solvent 1	Solvent 4	Solvent 5	Solvent 6 (ascending)	Solvent 7
HNI	0·98	0·93	0·90	0·98	0·92
HNI contaminant	0·98	0·84	0·77	0·94	0·69
n-Butylthiol	0·98	0·95	0·90	0·98	0·92
Coenzyme A	0·42	0·04	0·02	—	0·01
Cysteine	0·78	0·56	0·46	0·4	0·28
Cysteinylglycine	0·75	0·42	0·32	—	0·18
Glutathione	0·57	0·22	0·12	0·06	0·02
Homocysteine	0·84	0·57	0·46	—	0·31
Thioglycollate	0·95	0·74	0·71	0·28, 0·12	0·56
Thiomalate	0·90	0·63	0·62	—	0·29
Thioctic acid	0·98	0·90	0·90	0·6	0·86

with 10–20% (v/v) of water or when butanol was incorporated up to 15% (v/v) of the organic phase (Table 1). Such a system was also suitable for homocysteine, cysteinylglycine and coenzyme A. The non-amino acid thiols of low molecular weight move near the reagent in these solvent systems. For thioglycollic and thiomalic acids, solvent 5 and solvent 7 were more satisfactory. In these systems, however, glutathione sometimes became obscured, probably by interaction with carbonate.

The less polar thiol, thioctic acid, could be separated with solvent 6, but the resolution was not satisfactory. *n*-Butylthiol has thus far eluded separation from the reagent; it does, however, react as indicated by the rapid bleaching of the 'base-reaction' colour in the presence of excess of *n*-butylthiol.

As was reported by Tsou *et al.* (1955), HNI does not react with disulphides or thioethers. This was confirmed for cystine, djenkolic acid, thiourea and thiocoumarin.

The marker solutions of coenzyme A and cysteinylglycine gave multiple spots. The sample of coenzyme A included glutathione, cysteine, a trace of cysteinylglycine and three unidentified thiols running with R_F values of 0·27, 0·66 and 0·70 in solvent 4. (We are advised that current batches of Pabst coenzyme A are free of glutathione.) Cysteinylglycine marker solution also contained cysteine.

Sensitivity. Although 5 μm-mole quantities were convenient for exploratory studies, the dye formed by the reaction of HNI and TDA was sufficiently intense to be detected after development of the chromatogram down to 0·1 μm-mole. An experiment was performed in which decreasing quantities of marker solutions of cysteine and glutathione (30–0·1 μm-moles) in solvent 3 were applied on two halves of a filter-paper sheet. After development one half was dipped in TDA and the other in 0·25% ninhydrin (Gutcho & Laufer, 1954). In the first case all loci containing α-naphthol were stained including, of course, the reagent. It was apparent that, with the exception of cysteine, all levels of thiol could be detected with TDA; the lowest quantities of HNI–TDA were best perceived under ultraviolet light (366 mμ.) as bright-red fluorescent spots. With ninhydrin the sensitivity was three- to ten-fold less. Benesch *et al.* (1956) detected glutathione down to 33 μm-moles, a sensitivity 300-fold less than the present method.

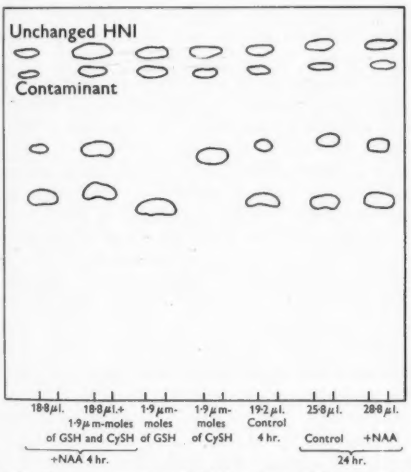


Fig. 2. Chromatography of thiols extracted from growing-pea-stem sections. Extracts were prepared as described in text from tissue floating on water or 50 μM NAA solution and harvested at various times. Extracts were chromatographed in solvent 2.

Applications to plant material. Inasmuch as this laboratory has been occupied with problems of plant growth, the applications of thiol chromatography have been for the most part restricted to studies with plant material. One analysis is presented here solely for the purpose of illustration.

Week-old etiolated pea stems were cut into 10 mm. subapical sections; 200-mg. lots were placed in Petri dishes containing 15 ml. of water or auxin solution 50 μM NAA and harvested at 4 and 24 hr. Extracts of the HNI derivatives of the free thiols were prepared as described above. The results are shown in Fig. 2.

Glutathione was found in amounts of the order of 0·1–0·3 μmole/g. fresh weight. Cysteine, although obscured by a light-pink substance present in the 'no-HNI' controls, was thought to be present at perhaps one-fifth of this amount. No other thiols were detected. Whereas differences between treatments with and without auxin could not be detected, a steady decline of thiols in both lots was evident over the 24 hr. growth period.

A more detailed report of the applications of this method for thiol analysis will be reported elsewhere (Price & Leopold, in preparation).

With all tissue preparations a 'no-HNI' control was essential, for a number of naturally occurring substances formed coloured products with TDA. This was particularly true of inflorescences and may have been due to the abundance of phenolic compounds related to anthocyanins. Ultraviolet light provides a further means for distinguishing HNI derivatives of thiols from other chromophores; only in the presence of HNI were any red-fluorescing materials observed.

SUMMARY

1. A method is described for the paper chromatography of thiols. The thiol is first treated with *N*-(4-hydroxy-1-naphthyl)*iso*maleimide and after

chromatographic development detected with tetrazotized di-*o*-anisidine.

2. The technique is applicable to a variety of thiols, but is not yet suitable for alkyl thiols.

3. Quantities of thiols as low as 0·3 μm-mole were detected, and under optimum conditions 0·1 μm-mole was visualized under ultraviolet light. The sensitivity of the method is therefore superior to the *N*-ethylmaleimide detection of thiols or the ninhydrin detection of 2-amino acids.

4. A sample application of the method is presented.

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The Synthesis of some Peptides Containing Glutamic Acid

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For use in experiments concerning the mechanism of the assimilation of glutamic acid by *Staphylococcus aureus* (Rowlands, Gale, Folkes & Marrian, 1957), a number of peptides containing glutamic acid have been prepared, of which L-valyl-L-glutamic acid, L-valyl-α-L-glutamyl-L-valine, L-leucyl-α-L-glutamyl-L-leucine and α-L-glutamyl-α-L-glutamyl-L-glutamic acid are new. α-L-Glutamyl-L-alanine has been prepared by an alternative route.

For the synthesis of L-valyl-α-L-glutamyl-L-valine, benzyloxycarbonyl-L-valine was converted into the mixed anhydride, with ethyl chloroformate in toluene solution, and this solution was allowed to react with an aqueous solution of the sodium salt of α-L-glutamyl-L-valine; the coupling product crystallized immediately, and hydrogenation gave the tripeptide. An analogous procedure succeeded in the preparation of L-leucyl-α-L-glutamyl-L-leucine. For the synthesis of α-L-glutamyl-α-L-glutamyl-L-glutamic acid, benzoyloxycarbonyl-L-glutamic anhydride was allowed to react with diethyl methyl α-L-glutamyl-L-glutamate; the isomeric products were separated by fractional extraction with aqueous sodium carbonate, and the α-isomer was hydrolysed and then hydrogenated to the tripeptide. The synthesis of L-valyl-L-glutamic acid requires no comment. α-L-Glutamyl-L-alanine was prepared in the normal fashion from benzyloxycarbonyl-L-glutamic anhydride (e.g. Le Quesne & Young, 1950*a*); its synthesis by a different route has since been reported by Sachs & Brand (1953).

The preparation of the γ-glutamyl peptides used in the work reported in the succeeding paper has been described previously (Le Quesne & Young, 1950*b*; Rowlands & Young, 1952), but recently Sachs & Brand (1954) have reported that the intermediate benzoyloxycarbonyl-γ-L-glutamyl azide may isomerize during coupling, to yield both α and γ products. We have therefore examined our original samples by paper chromatography with high concentrations of peptide and a mixture of *n*-butanol–acetic acid–water, as the mobile phase, and by comparison with samples to which known quantities of the α-isomer have been added we have estimated the approximate amount of the latter. The γ-L-glutamyl-glycine, -L-valine, -L-leucine, -L-tyrosine and -glycylglycine showed only traces (less than 0·2%) or none of the α-isomer. The γ-L-glutamyl-L-alanine was estimated to contain less than 0·5% and γ-L-glutamyl-L-glutamic acid less than 2% of the α-isomer, but the sample of γ-L-glutamyl-L-aspartic acid contained considerably more. In the synthesis of γ-L-glutamyl-L-glutamic acid only one of the intermediates after coupling

had been obtained crystalline, and in the synthesis of γ-L-glutamyl-L-aspartic acid both intermediates had remained as syrups which had evidently retained the α-isomer. It seemed possible that the benzyloxycarbonyl-γ-L-glutamylhydrazide used might still have contained some α-isomer; γ-ethyl benzyloxycarbonyl-L-glutamate was therefore recrystallized until hydrogenation gave a solution in which none of the α-ethyl isomer could be detected by paper chromatography; from this material, the hydrazide was prepared and recrystallized until hydrogenation gave a solution in which none of the α-hydrazide could be detected by paper chromatography. The benzyloxycarbonyl-γ-L-glutamylhydrazide so obtained was converted into the azide and coupled with L-alanine methyl ester and with diethyl L-glutamate. Without any purification, the products were saponified and hydrogenated, and the resulting solutions were examined by paper chromatography; in each case α-peptide was detected. Similarly, coupling of the azide with L-alanine benzyl ester gave a crude product which, after hydrogenation, was shown by paper chromatography to contain α-isomer. We therefore confirm the conclusion of Sachs & Brand (1954) that some isomerization of the γ-azide occurs, but when the subsequent products can be recrystallized this route would appear still to be useful for the preparation of γ-glutamyl peptides. γ-L-Glutamyl-L-glutamic acid has subsequently been prepared chromatographically pure by this method (Waley, 1955) and it is of interest that Abraham & Newton (1954) have prepared D-δ-amino-δ-carboxyvaleryl-glycine by this route; no isomeric peptide was found in the recrystallized product.

More recently we have attempted the synthesis of γ-L-glutamyl-L-aspartic acid by the method of Rudinger (1954) and of Swan & du Vigneaud (1954), in which toluene-*p*-sulphonyl-5-oxopyrrolidine-2-carboxylic acid is allowed to react with an amino ester. However, even under vigorous conditions diethyl L-aspartate gave a product which fractionation showed to be chiefly unchanged lactam, together with a small amount of coupling product (Winn & Young, unpublished work). Rudinger found that more drastic conditions were required for tyrosine ethyl ester than for glycine ethyl ester, and where such hindrance occurs the competitive formation of diketopiperazines is likely to cause reduced yields.

The determination of the partition coefficients of the peptides between *n*-butanol and water at 25° is described in the following paper.

EXPERIMENTAL

Melting points are uncorrected. The solvents used in paper chromatography were: *n*-butanol–acetic acid–water (62:12:26, by vol.), and phenol saturated with water. In the latter case the chromatography tank contained also 0·1% NH_3 soln. Before analysis, samples were dried over P_2O_5 at 15 mm. at 61° or 100° according to their melting points, but the results indicate that in some cases non-stoicheiometric water remained.

Benzyloxycarbonyl-L-valyl-α-L-glutamyl-L-valine. Benzyloxycarbonyl-L-valine (1·37 g.) and triethylamine (0·55 g.) were dissolved in toluene (20 ml.) and cooled to −5°. Ethyl chloroformate (0·59 g.) was added and the mixture was left in the freezing mixture for 5 min. α-L-Glutamyl-L-valine (1·34 g.) in N-NaOH (11 ml.) was then added and the mixture was stirred vigorously for 1 hr. at −5°. The freezing mixture was then removed and stirring continued for a further 2 hr. The aqueous phase was separated, washed with ether, and acidified with 5 N-HCl. The product crystallized immediately, and after being kept at 0° overnight it was filtered off, dried, and twice recrystallized from ethyl acetate–light petroleum. This gave *benzyloxycarbonyl-L-valyl-α-L-glutamyl-L-valine*, m.p. 245°, with previous sintering (1·5 g.; yield 58%). (Found: C, 56·5; H, 6·7; N, 9·1. $C_{23}H_{33}O_8N_3,\frac{1}{2}H_2O$ requires C, 56·6; H, 7·0; N, 8·6%.)

L-Valyl-α-L-glutamyl-L-valine. Benzyloxycarbonyl-L-valyl-α-L-glutamyl-L-valine (0·8 g.) was hydrogenated in the usual way in methanolic solution in the presence of palladium black, reduction taking 5–6 hr. The product readily solidified and was washed with ethanol and recrystallized from aqueous acetone. The *L-valyl-α-L-glutamyl-L-valine* (0·47 g.; yield 82%) had m.p. 225° with previous sintering; $[\alpha]_D^{19}$ −16·6° (*c*, 2·2 in 0·5 N-HCl). (Found: C, 47·7; H, 7·7; N, 10·6. $C_{15}H_{27}O_6N_3,2H_2O$ requires C, 47·3; H, 8·1; N, 11·0%.) R_F: in butanol–acetic acid–water, 0·56; in phenol–water, 0·69.

Benzyloxycarbonyl-L-leucyl-α-L-glutamyl-L-leucine. Benzyloxycarbonyl-L-leucine (1·7 g.) and triethylamine (0·65 g.) were dissolved in toluene (25 ml.) and cooled in a freezing mixture to −5°. Ethyl chloroformate (0·7 g.) was added, and then, after 5 min., α-L-glutamyl-L-leucine (1·67 g.) in N-NaOH (12·9 ml.). The mixture was stirred vigorously for 3 hr. whilst coming to room temperature. The aqueous phase was separated, washed with ether and acidified with 5 N-HCl. The product solidified immediately and had m.p. 101–103°. It was twice recrystallized from acetone–light petroleum, giving *benzyloxycarbonyl-L-leucyl-α-L-glutamyl-L-leucine*, m.p. 105° (1·6 g.; yield 43%). (Found: C, 58·5; H, 7·7; N, 8·3. $C_{25}H_{37}O_8N_3,\frac{1}{2}H_2O$ requires C, 58·1; H, 7·4; N, 8·1%.)

L-Leucyl-α-L-glutamyl-L-leucine. Benzyloxycarbonyl-L-leucyl-α-L-glutamyl-L-leucine (1·0 g.) was hydrogenated in the usual manner in aqueous methanol, reduction being complete in 3 hr. The product solidified immediately on evaporation, and was recrystallized from aqueous acetone, giving *L-leucyl-α-L-glutamyl-L-leucine* (0·55 g.; yield 75%), m.p. 228°; $[\alpha]_D^{19}$ −26·8° (*c*, 1·4 in 0·5 N-HCl). (Found: C, 51·8; H, 8·8; N, 10·3. $C_{17}H_{31}O_6N_3,H_2O$ requires C, 52·2; H, 8·4; N, 10·7%.) R_F: in butanol–acetic acid–water, 0·75; in phenol–water, 0·76.

Diethyl N-(benzyloxycarbonyl-α-L-glutamyl)-O-(methyl-α-L-glutamyl)-L-glutamate. Diethyl benzyloxycarbonyl-α-L-glutamyl-α-L-glutamate (Bergmann & Zervas, 1932) in ethyl acetate was converted into the methyl ester by the action of diazomethane in ether at 0°, and the product (7·5 g., m.p. 79–81°, after crystallization from ethanol–ether) was hydrogenated in a mixture of methanol (75 ml.) and N-HCl (20 ml.). After 2 hr. the theoretical quantity of carbon dioxide had been evolved. The solution was filtered

and evaporated to dryness *in vacuo* at room temperature, and the syrupy residue was evaporated several times with methanol to remove excess HCl. It was then coupled with benzyloxycarbonyl-L-glutamic anhydride as follows.

A solution of the hydrochloride (6·6 g.) and $KHCO_3$ (11 g.) in water (100 ml.), covered with ethyl acetate (100 ml.), was stirred mechanically at room temperature while a solution of benzyloxycarbonyl-L-glutamic anhydride (4·2 g.) in ethyl acetate (50 ml.) was added during 5–10 min.; stirring was continued for 5 hr. and the mixture was left overnight at 5°. The aqueous layer was separated, acidified with 5N-HCl and left for 1 hr. at 0°, when the solid material was filtered off and dried. It had m.p. 128–138° (3·9 g.; yield 41%). The solid was dissolved in ethyl acetate (50 ml.) and successively extracted with 20 ml. portions of 0·04N-Na_2CO_3. By refractionation of the later fractions and subsequent recrystallization from ethyl acetate–light petroleum diethyl *N*-(benzyloxycarbonyl-α-L-glutamyl)-*O*-(methyl-α-L-glutamyl)-L-glutamate was obtained (0·8 g.), m.p. 147–148°, $[\alpha]_D^{18}$ −33·3° (*c*, 1·5 in ethanol). (Found: C, 54·7; H, 6·0; N, 7·0. $C_{28}H_{39}O_{12}N_3$ requires C, 55·2; H, 6·4; N, 6·9%.) This material was saponified without further recrystallization.

Benzyloxycarbonyl-α-L-glutamyl-α-L-glutamyl-L-glutamic acid. Diethyl methyl benzyloxycarbonyl-α-L-glutamyl-α-L-glutamyl-L-glutamate (1·7 g.) was dissolved in N-NaOH (14 ml.) and allowed to stand for 2 hr. After acidification of the solution with 5N-HCl an oil was precipitated which crystallized during several days. Filtration, drying, and recrystallization from acetone yielded *benzyloxycarbonyl-α-L-glutamyl-α-L-glutamyl-L-glutamic acid* (1·2 g.; yield 82%) of m.p. 163–165°; $[\alpha]_D^{15}$ −17·1° (*c*, 1·3 in ethanol). (Found: C, 49·7; H, 5·8; N, 7·7. $C_{23}H_{29}O_{12}N_3,H_2O$ requires C, 49·6; H, 5·6; N, 7·5%.)

α-L-Glutamyl-α-L-glutamyl-L-glutamic acid. Benzyloxycarbonyl-α-L-glutamyl-α-L-glutamyl-L-glutamic acid (1·0 g.) in aqueous methanol containing a drop of acetic acid was hydrogenated in the usual way. After 3 hr. the theoretical quantity of carbon dioxide had been evolved. The solution was filtered and evaporated to dryness and the residue allowed to solidify under acetone, when it had m.p. 184–190°. It was twice reprecipitated from water with ethanol, giving *α-L-glutamyl-α-L-glutamyl-L-glutamic acid* (0·55 g.; yield 74%), m.p. 190–192°, $[\alpha]_D^{19}$ −7·2° (*c*, 1·44 in water). (Found: C, 44·7; H, 5·4; N, 10·5. $C_{15}H_{23}O_{10}N_3$ requires C, 44·4; H, 5·7; N, 10·4%.) R_P: in butanol–acetic acid–water, 0·24; in phenol–water, 0·03.

Benzyloxycarbonyl-L-valyl-L-glutamic acid. Benzyloxycarbonyl-L-valine (1·5 g.) was dissolved in ether (20 ml.) at 0° and PCl_5 (1·5 g.) was added, with stirring. After 15 min. a further amount of ether (10 ml.) was added, and the solution was quickly washed twice with ice–water (30 ml.). After drying (Na_2SO_4) briefly, the ether layer was mixed with a solution of diethyl L-glutamate hydrochloride (1·4 g.) and triethylamine (1·3 g.) in ethyl acetate (15 ml.). Next morning the solution was washed with dil. HCl, water, 5% $KHCO_3$ and finally water; it was then dried (Na_2SO_4) and evaporated to dryness *in vacuo*. The syrupy product failed to crystallize after being scratched and set aside for some days under light petroleum. It was dissolved in acetone (18 ml.) and added to N-NaOH (18 ml.) and left for 2 hr. at room temperature. The acetone was then removed by evaporation *in vacuo* below 30°, and the solution was acidified and extracted with several 10 ml. portions of ethyl acetate, the combined extracts being dried (Na_2SO_4) and evaporated *in vacuo*. The product crystallized after standing under light petroleum for some days. Several recrystallizations from ethyl acetate–light petroleum gave *benzyloxycarbonyl-L-valyl-L-glutamic acid* (1·1 g.; yield 49%), m.p. 105–106°. (Found: C, 56·9; H, 6·5; N, 7·4. $C_{18}H_{24}O_7N_2$ requires C, 56·8; H, 6·5; N, 7·4%.)

L-Valyl-L-glutamic acid. Benzyloxycarbonyl-L-valyl-L-glutamic acid (0·5 g.) was hydrogenated in the usual fashion, the evolution of carbon dioxide being complete after 7–8 hr. and several renewals of the catalyst. On evaporation of the filtered solution and recrystallization of the solid product from ethanol–water, *L-valyl-L-glutamic acid* was obtained (0·3 g.; yield 86%), m.p. 190–194°; $[\alpha]_D^{19}$ +3·2 (*c*, 1·5 in water). R_F: in butanol–acetic acid–water, 0·54; in phenol–water, 0·47. (Found: C, 49·1; H, 7·7; N, 11·2. $C_{10}H_{18}O_5N_2$ requires C, 48·8; H, 7·3; N, 11·4%.)

Benzyloxycarbonyl-α-L-glutamyl-L-alanine ethyl ester. A solution of L-alanine ethyl ester hydrochloride (4·6 g.) and $KHCO_3$ (8·3 g.) in water (50 ml.), covered with ethyl acetate (50 ml.), was stirred mechanically while a solution of benzyloxycarbonyl-L-glutamic anhydride (7·2 g.) in ethyl acetate (50 ml.) was added gradually during 2–3 min.; stirring was continued for 3 hr. The aqueous layer was then separated, washed with ether and acidified with 5N-HCl. The solid product was filtered off and dried. For the separation of the isomers it was dissolved in ethyl acetate (100 ml.) and extracted with 25 ml. portions of 1% Na_2CO_3, giving 10 fractions in all. The later fractions crystallized readily, the tenth having the highest m.p. (147–149°). The middle fractions were refractionated, combined with the last and recrystallized from ethyl acetate–light petroleum. This gave *benzyloxycarbonyl-α-L-glutamyl-L-alanine ethyl ester* (2·1 g.; yield 20%), m.p. 149–150°. (Found: C, 56·8; H, 6·3; N, 7·4. $C_{18}H_{24}O_7N_2$ requires C, 56·8; H, 6·5; N, 7·4%.)

Benzyloxycarbonyl-α-L-glutamyl-L-alanine. Benzyloxycarbonyl-α-L-glutamyl-L-alanine ethyl ester (0·6 g.) was dissolved in N-NaOH (4·8 ml.) and left for 2 hr. at room temperature. The solution was acidified with 5N-HCl and crystals separated on scratching. This gave *benzyloxycarbonyl-α-L-glutamyl-L-alanine* (0·5 g.; yield 90%), m.p. 176–177°, raised by recrystallization from acetone–ether to 178–179°. (Found: C, 54·4; H, 5·7; N, 8·1. $C_{16}H_{20}O_7N_2$ requires C, 54·5; H, 5·7; N, 8·0%.)

α-L-Glutamyl-L-alanine. Benzyloxycarbonyl-α-L-glutamyl-L-alanine (0·4 g.) was hydrogenated in the usual fashion. The product was recrystallized from aqueous ethanol, giving α-L-glutamyl-L-alanine (0·21 g.; yield 84%), m.p. 190°, $[\alpha]_D^{19}$ +10·1° (*c*, 1·4 in water). (Found: C, 43·7; H, 6·4; N, 12·8. Calc. for $C_8H_{14}O_5N_2$: C, 44·0; H, 6·4; N, 12·8%.) R_F: in butanol–acetic acid–water, 0·33; in phenol–water, 0·34.

Detection of α product from benzyloxycarbonyl-γ-L-glutamylhydrazide. The γ-ethyl benzyloxycarbonyl-L-glutamate used had m.p. 86°, and on hydrogenation gave a solution showing a single component when chromatographed on paper with butanol–acetic acid–water, which effects a good separation of α- and γ-ethyl glutamates. The hydrazide prepared from this ester was recrystallized from aqueous ethanol and finally from acetone–light petroleum, when it had m.p. 180°. On hydrogenation it gave a solution which showed a single component when chromatographed on paper with butanol–acetic acid–water, which separates α-

and γ-glutamylhydrazides effectively. The pure hydrazide was converted into the azide in chloroform solution and coupled in one experiment with L-alanine methyl ester and in another with diethyl L-glutamate, according to the procedure of Le Quesne & Young (1950*b*). The products were saponified with N-NaOH, and after acidification the acids were extracted into ethyl acetate, which, after drying (Na_2SO_4), was removed under reduced pressure. The crude residues were hydrogenated in the normal fashion, with palladium black as catalyst, in the presence of acetic acid. The crude products (10% solutions in water) were examined by paper chromatography, with butanol–acetic acid–water as solvent; in both cases α-peptide was detected. A further portion of the pure hydrazide was coupled with L-alanine benzyl ester under the conditions of Sachs & Brand (1954); hydrogenation of the crude product gave material which, without purification, was examined by paper chromatography as above, and again α-isomer was detected.

SUMMARY

1. For use in experiments concerning the mechanism of the assimilation of glutamic acid by *Staphylococcus aureus* (Rowlands *et al.* 1957), the following new peptides have been synthesized: L-valyl-L-glutamic acid, L-valyl-α-L-glutamyl-L-valine, L-leucyl-α-L-glutamyl-L-leucine, α-L-glutamyl-α-L-glutamyl-L-glutamic acid. α-L-Glutamyl-L-alanine has been prepared by an alternative route.

2. When γ-glutamyl peptides are synthesized through benzyloxycarbonyl-γ-L-glutamyl azide some α products may occur in the crude material (as found by Sachs & Brand, 1954), but subsequent recrystallization will usually eliminate the α-isomer.

Analyses were by Weiler and Strauss, Oxford. We are grateful to the Medical Research Council for a grant for scientific assistance.

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The Assimilation of Amino Acids by Bacteria

23. ACCUMULATION OF FREE GLUTAMIC ACID WITHIN *STAPHYLOCOCCUS AUREUS* INCUBATED WITH DERIVATIVES OF GLUTAMIC ACID*

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Gram-positive bacteria concentrate certain amino acids in the free state in the internal medium of the cells (Gale, 1947, 1953; Taylor, 1947). The passage of amino acids across the cell wall and associated structures may be by a process of diffusion, as with lysine (Gale, 1947; Najjar & Gale, 1950), or by an active process involving exergonic metabolism on the part of the cell, as with glutamic acid (Gale, 1947). If washed suspensions of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) are incubated with glucose and glutamic acid, the amino acid enters the cell and becomes concentrated therein, the ratio of internal to external glutamic acid becoming as high as 350–400:1 when the external concentration is small. The accumulation is prevented by the presence of inhibitors of fermentation, 'uncoupling agents', 8-hydroxyquinoline, or by growth of the cells in the presence of penicillin for a short time before harvesting (Gale, 1949, 1951; Gale & Taylor, 1947). Free amino acids accumulate

* Part 22: Creaser (1956).

within the cells when protein synthesis is inhibited or when cells are incubated in media containing single amino acids or simple mixtures which are unable to support protein synthesis (Gale, 1953).

Understanding of the relation between amino acid accumulation and protein synthesis, and of the importance of the accumulation process to the life of the cell, will involve elucidation of the energy-linked process concerned in the active systems. It may be that there is a membrane which lies in the surface of *Staphylococcus* and is impermeable to glutamic acid, passage of this amino acid into the cell occurring by active transport. Alternatively, it may be that the surface structures are permeable to glutamic acid and that the substance measured within the cell as 'free glutamic acid' is actually a labile derivative thereof which cannot be distinguished from the free amino acid by present methods of detection and liberation from the cell. Britten (1952) investigated the passage of ^{14}C-labelled glutamic acid into *Staph. aureus* and distinguished two processes: a temperature-dependent exchange between added glutamic acid and glutamic acid 'stores' within the cells, and a rapid temperature-independent exchange between added amino acid and glutamic acid in a 'water space' corresponding to 30–40 % of the packed cell (pellet) volume. This latter finding appeared to support the hypothesis that glutamic acid diffuses freely into a substantial portion of the staphylococcal cell, but further investigation (Britten, 1955) showed that the 'water space' included intercellular water corresponding to 26–30 % of the pellet volume and that, consequently, the staphylococcal cell itself was impermeable to free glutamic acid. This fact has been confirmed by Mitchell & Moyle (1956), who have established the existence of an osmotic barrier in this organism. The present communication deals with the accumulation of free glutamic acid within staphylococci incubated in media containing various derivatives of glutamic acid.

ORGANISM AND METHODS

Organism. The organism used throughout this work was the strain of *Staph. aureus* Duncan used for earlier studies of amino acid assimilation (Gale, 1947, 1951) and also used by Britten (1952). Cultures were grown for 14 hr. at 25° in Roux bottles, each containing 150 ml. of the deficient medium previously described (salt solution containing arginine, Marmite and glucose; Gale, 1947). Cells were harvested on the centrifuge, washed once and suspended in water; the suspension density was determined turbidimetrically in a Hilger absorptiometer previously calibrated against the organism used.

Incubation conditions. Centrifuge tubes (100 ml.) were incubated in a water bath at 37° (or other temperature as indicated); each tube contained 20 ml. of buffered saline solution, pH 7·0 (Gale, 1947), 1·0 ml. of 25 % (w/v) glucose, 1·5 ml. of solution of glutamic acid derivative under test and 2·5 ml. of suspension of washed *Staph. aureus* adjusted to a suspension density of 20 mg. dry wt. of organisms/ml. In controls in which components of the incubation mixture were missing, the volume was adjusted to 25 ml. with water. Incubation was stopped when desired by addition to each tube of 10 ml. of 0·2 M acetate buffer, pH 4·5, and about 15 g. of crushed ice. Organisms were then centrifuged down, washed twice in 0·01 M acetate buffer, pH 4·5, made up to 2·5 ml. in water, and the free glutamic acid within the cells was determined by the specific decarboxylase method (Gale, 1945, 1947).

Rate of accumulation of free glutamic acid within cells incubated with derivatives. The rate of accumulation of free glutamic acid within the cells is dependent upon the external concentration of amino acid for low values of the latter, but independent once the external concentration has exceeded a saturation value (Gale, 1947). It was desirable in these experiments to work at concentrations of derivatives exceeding the saturation level, although the limited supply of some of the substances made it impossible to use these in concentrations well in excess of the saturation value. Consequently, preliminary experiments were carried out with each derivative in order to determine the saturation concentration; with glutamyl peptides this concentration was approx. 1·0 μmole/ml., and later experiments were carried out in a final concentration of 1·5–2·0 μmoles of peptide/ml. Experiments were then performed in which the rate of accumulation of free glutamic acid within the cells was determined during incubation with the derivative in the presence and absence of glucose; in all cases the results were compared with the rate of accumulation occurring in cells incubated in the presence of glutamic acid and glucose. In some studies the effect of temperature on the process was investigated by carrying out incubations simultaneously at 20°, 25° and 37°.

Determination of rate of hydrolysis of peptides within cells. As Zamecnik & Stephenson (1947) have shown, the rate of hydrolysis of a peptide can be followed in the presence of the specific amino acid decarboxylase which attacks one of the hydrolysis products. Glutamic acid decarboxylase does not attack peptides containing glutamic acid (Gale, 1946) and can be used to study the rate of liberation of glutamic acid from peptides. To obtain a measure of the rates of hydrolysis, within *Staph. aureus* cells, of the peptides used in these studies, the following procedure was adopted: a thick suspension of cells in 0·1 M phosphate buffer, pH 5·5, was disintegrated by exposure to supersonic vibration at 25 kcyc./sec. for 30 min. in a Mullard 500 w magnetostrictor oscillator, and 1·0 ml. of the disintegrated suspension (approx. 30 mg. dry wt. of cells) was shaken in a Warburg vessel with 1·0 ml. of L-glutamic acid decarboxylase preparation (Gale, 1945), 1·0 ml. of 0·2 M acetate buffer, pH 5·5, and 0·5 ml. of 0·035 M peptide, the peptide and decarboxylase preparations being made up in acetate buffer and adjusted to pH 5·5. The steady rate of CO_2 evolution was taken as a measure of the liberation of glutamic acid from the peptide; the amount of decarboxylase preparation used was such that the rate of decarboxylation of glutamic acid was 2–3 times greater than the fastest liberation of CO_2 from the system containing peptide preparations.

Determination of partition coefficients. A quantity (10 μmoles) of each peptide was weighed out and dissolved in 1·0 ml. of water saturated with *n*-butanol. The peptide

solution was placed in a 10 ml. tube and 5·0 ml. of *n*-butanol saturated with water added; the tube was sealed and shaken overnight at room temperature (20–22°) and then left at 25° for 48 hr. For estimation, 0·1 ml. of the aqueous phase and 1·0 ml. of the butanol phase were withdrawn, run into small beakers and evaporated to dryness over $CaCl_2$. The remaining mixture was transferred to a new tube, washed in with 0·1–0·3 ml. of water, saturated with butanol, resealed and equilibrated as above. Further samples were then withdrawn for estimation and the whole operation was repeated so that three consecutive estimations of the partition coefficient were obtained. The amount of peptide in each phase was estimated by development of colour with ninhydrin according to the procedure of Troll & Cannan (1953); the material in the evaporated butanol phase was dissolved in 0·17 M sodium acetate solution (0·5 ml.), 0·2 ml. of ninhydrin and 1·0 ml. each of the phenol and KCN–pyridine solutions were added and the solution was washed into a heating tube with two 0·5 ml. quantities of 60% ethanol, heated for 4 min. at 100° and finally made up to 10·0 ml. with 60% ethanol. The equivalent material from the aqueous phase was treated with twice these quantities of the various reagents and the final volume adjusted to 20·0 ml. The optical density of the solutions was compared at 570 mμ. and estimated against standard peptide solutions.

Sources of glutamic acid derivatives

We are indebted to Dr J. S. Fruton for samples of α-L-glutamyl-L-glutamic acid, to Dr A. C. Chibnall for β-hydroxyglutamic acid, 5-oxo-L-pyrrolidine-2-carboxylic acid and α-aminobutyric acid, and to Dr D. W. Clayton for diethyl L-glutamate, α-L-glutamyl-L-glutamic acid and α-L-glutaminyl-L-glutamic acid.

N-Phosphoryl-L-glutamic acid was prepared as the dimagnesium salt by the method of Winnick & Scott (1947). Its glutamic acid content was determined by estimation of the free glutamic acid liberated after 10 min. hydrolysis at 100° in N-HCl. Incubation of *N*-phosphoryl-L-glutamic acid with glutamic acid decarboxylase resulted in a slow evolution of CO_2 corresponding to the rate of liberation of free glutamic acid by hydrolysis.

N-Acetyl-L-glutamic acid was prepared by the method of Cahill & Burton (1940). This substance was not attacked by glutamic acid decarboxylase.

Diethyl L-glutamate hydrochloride was prepared by the method of Chiles & Noyes (1922); it was not attacked by glutamic acid decarboxylase.

Diethyl N-dibenzylphosphoryl-L-glutamate was prepared by the general procedure reported by Atherton, Openshaw & Todd (1945). Diethyl L-glutamate (5·54 g.) and 7·15 g. of dibenzyl phosphite were dissolved in CCl_4 (25 ml.) and drops of a solution containing 2·8 ml. of *N*-methylmorpholine in 5 ml. of CCl_4 added. A precipitate formed and the whole was left overnight at room temperature. An equal volume of water was then added and the organic layer separated. The aqueous phase was extracted three times with 10 ml. of CCl_4 and the combined extracts were added to the original layer and dried over anhydrous Na_2SO_4. Removal of the solvent *in vacuo* left an almost colourless residue which rapidly crystallized. The solid was dissolved in CCl_4 (15 ml.), filtered and treated with 25 ml. of dry hexane. On standing, colourless needles of diethyl *N*-dibenzylphosphoryl-L-glutamate separated, m.p. 66–67°, yield 60·5%. (Found: C, 59·6; H, 6·69; N, 2·72; calc. for $C_{23}H_{30}O_7NP$: C, 59·6; H, 6·53; N, 3·02%.)

Diethyl N-phosphoryl-L-glutamate. Diethyl *N*-dibenzylphosphoryl-L-glutamate (3·0 g.) was dissolved in 100 ml. of 95% ethanol and hydrogenated over reduced palladium oxide (0·3 g.) at room temperature and pressure. When 318 ml. of H_2 had been absorbed, uptake ceased (removal of two benzyl groups requires 290 ml. at s.t.p.). The catalyst was filtered off and the solvent removed *in vacuo* at 30°, leaving a clear resin (1·85 g.; yield 100%). (Found: C, 38·6; H, 6·49; N, 4·66; calculated for $C_9H_{18}O_7NP$: C, 38·1; H, 6·35; N, 4·95%.) The compound was neutralized before testing.

Glutamyl peptides. The preparation of peptides, other than those mentioned above, is described in the preceding paper (Rowlands & Young, 1957).

RESULTS

Substances giving rise to free glutamic acid within cells incubated in the absence of glucose

N-Phosphoryl-L-glutamic acid. Fig. 1 shows the time course of the accumulation of free glutamic acid within *Staph. aureus* incubated with *N*-phosphorylglutamic acid in the presence and absence of glucose. In the presence of glucose, the accumulation within the cell is the same whether

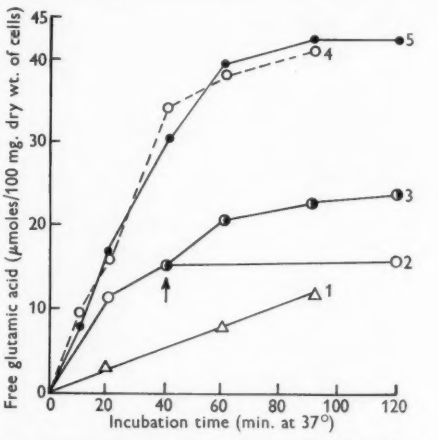

Fig. 1. Accumulation of free glutamic acid within *Staph. aureus* incubated with various external sources of glutamic acid. Cells were incubated at a final suspension density of 2·0 mg. dry wt./ml. in buffered saline at pH 7·0 (glutamic acid, diethyl glutamate) or 5·5 (*N*-phosphorylglutamate), at 37° in the presence of the following: curve 1, 9 μmoles of diethyl L-glutamate/ml.; curve 2, 9 μmoles of *N*-phosphoryl-L-glutamate/ml. added at time 0; curve 3, as for curve 2 with a further 9 μmoles of *N*-phosphorylglutamate/ml. added at time 40 min. as indicated by arrow; curve 4, as for curve 2, with addition of 1% (w/v) of glucose; curve 5, 5 μmoles of L-glutamate and 1% of glucose.

glutamic acid itself or the *N*-phosphoryl derivative is the external source. In the absence of glucose, no accumulation occurs when glutamic acid is the external source but a significant accumulation occurs when *N*-phosphorylglutamic acid is present in the external medium. The rate of accumulation in the latter case varied widely in different experiments but has reached values 60–70 % of that obtained when glucose and glutamic acid are present. The figure shows that the accumulation with *N*-phosphorylglutamic acid as source rapidly decreases in rate. The phosphoryl derivative is unstable and it seemed probable that the rapid cessation of glutamic acid accumulation might be due to decomposition of the derivative in the medium during incubation; that this is the explanation is shown by the fact that accumulation is resumed if a further supply of phosphoryl derivative is added to the incubation medium, as shown in Fig. 1. The nature of the progress curve after a single addition makes it impossible to carry out an accurate study of the kinetics of the process, and conclusions have to be based upon rates measured within 20 min. of the addition. Determination of the rate of accumulation over a range of temperatures gives a value of approx. 2·4 for the temperature coefficient. Fig. 2 shows that the optimum pH for glutamic acid accumulation in this case is approx. 5·5.

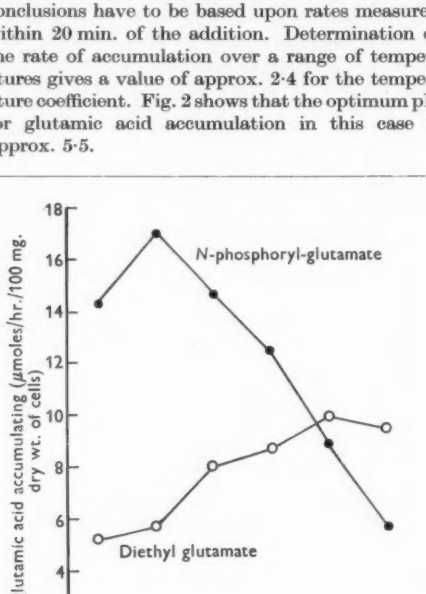

Fig. 2. Effect of pH of medium on rate of accumulation of free glutamic acid within *Staph. aureus* incubated with either *N*-phosphoryl-L-glutamate or diethyl L-glutamate. Conditions were as for curve 1 or 2 of Fig. 1.

Diethyl L-*glutamate*. Fig. 1 shows that a slow accumulation of free glutamic acid takes place in *Staph. aureus* incubated in the presence of diethyl glutamate. The further addition of glucose was found usually to have little effect; in some experiments the rate of accumulation was increased by approx. 50 % in the presence of glucose, whereas in the majority of tests the enhancement was within experimental error. Fig. 2 shows that the optimum pH for the accumulation was 8·5, although the effect of pH was not marked over the range tested. The temperature coefficient for the process over the range 20–37° was 1·8; this is the lowest coefficient measured in the present experiments.

The accumulation of free glutamic acid within cells incubated with diethyl glutamate must follow upon two processes: passage of the ester or its hydrolysis products into the cell, and hydrolysis of the ester to liberate free glutamic acid. It seemed possible that the latter process might be limiting. To test this, cells were incubated with ester in the usual way, centrifuged down and the cell batch divided into two; the free glutamic acid content of one portion was determined immediately while the other portion was washed and then incubated for 1 hr. at 37° in the absence of added ester before determination of the free glutamic acid content. Table 1 shows that there is a marked increase in the free glutamic acid content during the second period of incubation, presumably due to hydrolysis of ester accumulated (but not hydrolysed) within the cell during the first incubation period. It would appear, therefore, that the rate of penetration of ester into the cells is greater than its rate of hydrolysis to the free acid within the cells.

Table 1. *Accumulation of free glutamic acid within* Staphylococcus aureus *incubated with diethyl glutamate*

Washed suspension of *Staphylococcus aureus* Duncan was incubated at a final density of 2·2 mg. dry wt. of cells/ml. in buffered saline, pH 7·5, containing 9·0 μmoles of diethyl glutamate/ml. at 37°; samples were withdrawn at 30 and 60 min. and divided into two: one portion was analysed immediately for free glutamic acid, and the second portion was washed once in water, suspended in buffered saline at 2·2 mg. dry wt./ml. and incubated for 1 hr. at 37° before analysis for free glutamic acid.

Time of first incubation (min.)	Increase in internal free glutamic acid (μmoles/100 mg. dry wt. of cells): Estimated on first harvesting	Estimated after second incubation
30	2·3	9·25
60	6·9	22·1

Diethyl N-phosphoryl-L-glutamate. *Staph. aureus* cells were incubated with a range of concentrations of a preparation of diethyl *N*-phosphorylglutamate in the presence and absence of glucose over the pH range 5·0–9·0. In no case was there any significant accumulation of free glutamic acid within the cells.

Substances giving rise to free glutamic acid within cells incubated in the presence of glucose

Glutamine. Fig. 3 shows the course of accumulation of free glutamic acid within *Staph. aureus* incubated with glutamine and glucose. The rate of accumulation is slower than that obtained when free glutamic acid itself is present in the external medium. No accumulation takes place if glucose is omitted from the medium.

Peptides containing a glutamic acid residue. Table 2 lists the glutamyl peptides tested which give rise to free glutamic acid within cells when incubation takes place in the presence of glucose; no peptide has yet been obtained which will give rise to free glutamic acid within cells in the absence of glucose in the incubation medium. The rate at which free glutamic acid accumulates (expressed in all cases in terms of the rate with glutamic acid and glucose present as 100) within the cells varies widely with the peptide used as source. In general, α-glutamyl peptides are markedly better sources than the corresponding γ-glutamyl peptides. Fig. 3 shows the time course of the accumulation that occurs when various polyglutamic acid peptides are used as source; the substances have the following order of decreasing efficiency as source: glutamic acid, α-glutaminylglutamic acid, α-glutamylglutamic acid, γ-glutamylglutamic acid, α-glutamyl-α-glutamylglutamic acid. In all cases the sources used consisted of L-isomers only. α-Glutamylglycine is almost as effective a source as glutamic acid itself, and both α-glutamylvaline and α-glutamylleucine give rise to glutamic acid within the cell at rates markedly greater than that obtained with glutamic acid itself as external source.

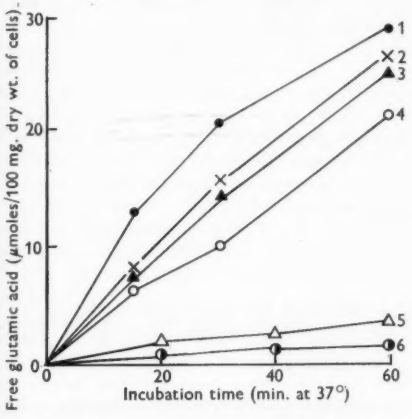

Fig. 3. Rate of accumulation of free glutamic acid within *Staph. aureus* incubated with various external sources of glutamic acid. Cells were incubated at a final suspension density of 2·0 mg. dry wt./ml. in buffered saline at pH 7·0 containing 1% (w/v) of glucose and the following sources of glutamic acid at a final concentration of 1·5 μmoles/ml.: curve 1, sodium L-glutamate; curve 2, α-L-glutaminyl-L-glutamic acid; curve 3, L-glutamine; curve 4, α-L-glutamyl-L-glutamic acid; curve 5, γ-L-glutamyl-L-glutamic acid; curve 6, α-L-glutamyl-α-L-glutamyl-L-glutamic acid.

Factors affecting the rate of accumulation. Inspection of the data in Table 2 suggests that two factors might influence the rate of accumulation: (1) since peptides containing valine or leucine are more effective sources than those containing less lipid-soluble residues, it seems possible that penetration of a lipid-like barrier affects the rate of accumulation within the cell; (2) since α-glutamyl peptides are more effective sources than γ-glutamyl peptides, it seems possible that the action of an α-peptidase is limiting in some cases. As a measure of the lipid solubility of the derivatives, the partition coefficient between *n*-butanol and water was determined, and the peptidase action of a broken cell preparation (broken cells being used to avoid complications due to differences in penetration) towards various peptides was measured as described above. The results are shown in Table 2, and it can be seen that there are wide variations both in the rates of hydrolysis and in the partition coefficients of the peptides tested. In certain cases (e.g. α-glutamylglutamic acid, α-glutaminylglutamic acid, α-glutamyl-tyrosine or -leucine) there appears to be correspondence between high rates of accumulation and high partition coefficients, but there is no such correlation for the whole range of peptides studied. Likewise there are some examples, such as alanylglutamic acid and α-glutamyl-valine or -leucine, where high rates of accumulation correspond with rapid rates of hydrolysis, but this correspondence does not extend to the tripeptides studied. However, there is a highly significant correlation between the rate of accumulation and the product of the partition coefficient and the rate of hydrolysis.

The rates of hydrolysis of γ-glutamyl peptides are within the experimental error of the method used and, although such peptides are poor sources of internal free glutamic acid, there would nevertheless appear to be a correlation between their activity in this sense and their partition coefficient.

Table 2. *Rate of accumulation of free glutamic acid within* Staphylococcus aureus *incubated in the presence of glucose and peptides containing glutamic acid*

Rate of accumulation was determined in washed suspensions of cells incubated at a final density of 2·0 mg. dry wt./ml. in buffered saline, pH 6·5, containing 1% of glucose and peptide at final concn. of 1·5 μmoles/ml. Temperature, 37°. Rates are expressed on basis of rate obtained with L-glutamic acid as source = 100. Temperature coefficients are calculated as increase in rate on raising temperature from 20 to 30°. Partition coefficients are between *n*-butanol and water, and rates of hydrolysis are determined as in text; rate of hydrolysis is expressed as μl. of CO_2 evolved/hr. from system containing acetate buffer, pH 5·5, 0·005M peptide, disintegrated *Staph. aureus* and L-glutamic acid decarboxylase preparation.

Peptide	Rate of accumulation of free glutamic acid (*a*)	Temp. coefficient (*b*)	Partition coefficient (butanol–water) (*c*)	Rate of hydrolysis (*d*)	(*c* × *d*)
α-L-Glutamyl-L-leucine	180	2·33	0·08	1500	120
α-L-Glutamyl-L-valine	140	2·25	0·046	1300	60
L-Leucyl-α-L-glutamyl-L-leucine	115	—	0·18	288	52
L-Alanyl-L-glutamic acid	110	—	0·024	1110	27
L-Valyl-L-glutamic acid	106	—	0·044	1100	49
(L-Glutamic acid)	100	2·8	0·027	—	—
α-L-Glutamyl-L-alanine	100	—	0·013	1010	13
L-Valyl-α-L-glutamyl-L-valine	94	—	0·076	377	29
α-L-Glutamyl-L-tyrosine	89	—	0·06	920	55
α-L-Glutamylglycine	88	—	—	—	—
α-L-Glutaminyl-L-glutamic acid	69	2·5	0·035	565	20
(L-Glutamine)	60	2·5	0·021	—	—
α-L-Glutamyl-L-glutamic acid	45	2·0	0·013	720	9·4
α-L-Glutamyl-L-aspartic acid	35	—	0·017	813	14
Glycyl-L-glutamic acid	22	—	—	—	—
γ-L-Glutamyl-L-valine	20	—	0·11	—	—
γ-L-Glutamyl-L-leucine	16	—	0·21	—	—
Glutathione	15	2·5	—	—	—
γ-L-Glutamyl-L-glutamic acid	13	2·4	0·025	3	—
α-L-Glutamyl-α-L-glutamyl-L-glutamic acid	7	—	0·015	435	6·5
γ-L-Glutamyl-L-aspartic acid	5	—	0·018	0	—
γ-L-Glutamylglycine	3	—	0·021	—	—

Statistical data

α-Glutamyl peptides

Correlation between first and last columns:

$(c \times d) = 0{\cdot}57(a) - 14$,
$N = 12$, correlation coeff. = 0·845, $P < 0{\cdot}01$.

γ-Glutamyl peptides

Correlation between first and third columns:

$c = 0{\cdot}0102(a) - 0{\cdot}040$,
$N = 5$, correlation coeff. = 0·944, $P = 0{\cdot}02$.

Table 3. *Concentration of inhibitors producing* 50% *inhibition of the rate of accumulation of free glutamic acid within* Staphylococcus aureus *incubated with glutamic acid derivatives*

Conditions as in Fig. 1 and Table 2.

Derivative	Sodium azide (mM)	2:4-Dinitrophenol (mM)	8-Hydroxyquinoline (mM)
A. In the presence of glucose			
L-Glutamic acid	6·4	0·45	0·08
L-Glutamine	9·0	0·40	0·08
α-L-Glutamyl-L-glutamic acid	3·5	0·24	—
α-L-Glutamyl-L-valine	—	0·20	—
Glutathione	2·0	0·08	1·2
(Glucose fermentation)	10	10	5·0
B. In the absence of glucose			
N-Phosphoryl-L-glutamic acid	3·2	0·3	2·0
Diethyl L-glutamate	6·4	0·3	5·0

Actions of inhibitors on the accumulation of glutamic acid within cells incubated with various external sources

Table 3 shows the concentration of certain metabolic inhibitors which decreases by 50 % the rate of accumulation of glutamic acid within cells incubated with various external sources of the amino acid. The inhibition obtained when glutamic acid is the external source has been discussed in previous papers (Gale, 1949, 1951). There are no marked differences in the sensitivities of the accumulation processes with the various sources in the presence of either sodium azide or 2:4-dinitrophenol, although the accumulation occurring in the presence of *N*-phosphorylglutamic acid or of diethyl glutamate (in the absence of glucose) is much less sensitive to the action of 8-hydroxyquinoline than that which occurs when glutamic acid, in the presence of glucose, is the external source.

DISCUSSION

Argument concerning the nature of the active concentration of free glutamic acid within *Staph. aureus* centres on the question whether energy is required to transport glutamic acid across an otherwise impermeable membrane, or whether it is required for some metabolic modification or binding after diffusion of the molecule into the cell interior. When a derivative of glutamic acid is used as the external source, accumulation of the free amino acid within the cell will depend also upon liberation of glutamic acid from the derivative. From a study of the factors involved in controlling the rate of accumulation of free glutamic acid within cells incubated with various derivatives thereof, it should be possible to decide whether there is any barrier to free penetration of any of these derivatives, since such a barrier would impose a limitation on their accumulation and metabolism within the cell.

When glutamyl peptides are supplied as external source, the rate of accumulation of free glutamic acid within the cell can be correlated with the product of the partition coefficient between *n*-butanol and water, and the rate of hydrolysis of the peptide by cell extracts. If no penetration factor were involved, a correlation would be expected with the rate of hydrolysis alone. The penetration factor might relate either to the peptide itself or to the products of hydrolysis, assuming this to take place before penetration. Since the same product of hydrolysis, glutamic acid, is estimated in all cases, it follows that the penetration factor must relate to the peptide and not to the products. It seems probable, therefore, that the various peptides which give rise to free glutamic acid within the cells have to penetrate some barrier before they are broken down to liberate free glutamic acid. That external glutamic acid itself has to pass some barrier is indicated by the fact that certain peptides, such as α-glutamyl-valine and -leucine, give rise to a more rapid accumulation of glutamic acid within the cell than is obtained with glutamic acid itself as external source. This confirms the demonstration by Mitchell & Moyle (1956) that the staphylococcal cell is impermeable to free glutamic acid. The presence of a barrier is further indicated with diethyl glutamate, where evidence has been obtained of accumulation of the ester within the cell, the ester remaining within the cell despite washing with water.

The only substances which have been found to yield glutamic acid within the cell when incubation has taken place in the absence of glucose have been the diethyl ester and the *N*-phosphoryl derivative of glutamic acid. If the temperature coefficient can be taken as any indication of the energy of activation in a system as complex as this, the ester passes into the cell more readily than the phosphorylated derivative. In all the other cases investigated, a source of energy is necessary for the accumulation of free glutamic acid, which suggests that a process of active transport across the barrier is involved. It is possible that the breakdown of some molecules of *N*-phosphorylglutamic acid yield energy for the transport of other molecules or of the liberated glutamic acid; this would be in accord with the high temperature coefficient for the overall process and also with its sensitivity to uncoupling agents such as sodium azide and dinitrophenol. It seems probable that the penetration of the ester occurs by passive transport, although the overall process is again sensitive to sodium azide and dinitrophenol. Cohen & Rickenberg (1956) have recently demonstrated that the passage of amino acids into *Escherichia coli* is an energy-linked reaction and have suggested that enzyme-like 'permeases' in the cell membrane are involved.

Kihara, McCullough & Snell (1952) have shown that D-alanine prevents the growth of *Lactobacillus arabinosus* by inhibiting the assimilation of L-alanine, but that D-alanine has no inhibitory action in the presence of alanyl peptides. The authors suggest that the D-amino acid inhibits the passage of L-alanine across the cell membranes, whereas peptides pass independently and are converted into the free amino acids within the cell. A similar hypothesis has been developed to explain other instances where the inhibitory action of amino acid analogues is overcome by the presence of peptides (Kihara & Snell, 1955). The results presented in this communication are consistent with such hypotheses.

SUMMARY

1. Free glutamic acid accumulates within *Staphylococcus aureus* incubated in the presence of *N*-phosphoryl-L-glutamic acid or diethyl glutamate.

2. Free glutamic acid does not accumulate within cells incubated with peptides containing glutamic acid residues unless glucose is also present. The rate of accumulation is higher when incubation takes place with α-glutamyl peptides than with γ-glutamyl peptides and is affected by the rate of hydrolysis of the peptide within the cell and also by the partition coefficient of the peptide between *n*-butanol and water.

3. When *Staphylococcus aureus* is incubated with glucose and di- or tri-peptides containing α-glutamyl residues, the rate of accumulation of free glutamic acid within the cells can be correlated with the product of the rate of hydrolysis and the partition coefficient between butanol and water.

4. Accumulation of free glutamic acid in all cases studied is inhibited by sodium azide and 2:4-dinitrophenol.

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Phospholipids

4. ON THE COMPOSITION OF HEN'S EGG PHOSPHOLIPIDS*

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Despite the long-established use of egg yolk as a convenient source of phospholipids, data available in the literature on the composition of this important fraction of the yolk (over 20% of the total solids) are incomplete and contradictory. Wittcoff (1951), in his recent monograph, was able to quote only three analyses, the earliest of which (30% lecithin, 70% kephalin) is clearly in conflict with the later data of Nottbohm & Mayer (1933) (72% lecithin, 28% kephalin) and of Kaucher, Galbraith, Button & Williams (1943) (72% lecithin, 25% kephalin, 2·5% sphingomyelin).

The contents of lecithin obtained by these later workers were based upon estimation of choline after hydrolysis, as were the recently published figures of 80% (van Handel, 1954), 82% (Sulser, 1954), 76% (Thiele, 1955) and 78% (Tsuji, Brin & Williams, 1955).

The remainder of the lipid nitrogen is usually reported as 'kephalin', but direct determination of the amino nitrogen has indicated considerably higher values, e.g. 42% (Chargaff, Ziff & Rittenberg, 1942), 38% (Kline & Johnson, 1946), 35% (Kline, Gegg & Sonoda, 1951) and 9·4, 20 and 32% (Sulser, 1954). Chargaff *et al.* (1942) used a direct van Slyke determination on the intact phospholipid, a procedure known to be subject to interference by the unsaturated acids present (Lea & Rhodes, 1954). Kline *et al.* (1951) also used this method, but stated that the chosen reaction time of 5 min. gave a

* Part 3: Lea, Rhodes & Stoll (1955).

result agreeing with a similar determination on a hydrolysate. Sulser (1954) separated the water-soluble hydrolysis products by paper chromatography and obtained the quoted figures by three different methods of estimating the recovered ethanolamine.

Any method using amino nitrogen content as a measure of 'kephalin' is liable to give too high values unless the lipid preparation has been freed from amino acids, which are tenaciously held (Lea & Rhodes, 1953); such errors can be considerable since the nitrogen content of a diacylglycerophospholipid is less than 2 %. Our own recently determined values of amino nitrogen/phosphorus or amino nitrogen/total nitrogen for samples of the acetone-insoluble phospholipids of egg, from which water-soluble contaminants had been removed, were much lower than those of the American workers, e.g. 21 % (Lea & Rhodes, 1953), 19 % (Lea & Rhodes, 1954) and 19 % (Lea & Rhodes, 1955).

Part of the wide variation in the proportions recorded for the major constituents of the phospholipids of egg may well, therefore, be due to the unsatisfactory nature of the analytical methods which have been used. A further factor could be the artificial concentration of one component by the purification procedures employed; the phospholipid preparation examined by Chargaff *et al.* (1942), for example, contained less than one-half of the average lipid phosphorus of the egg.

Small amounts of sphingomyelin appear to be present in egg, although Schmidt (1946) and Tsuji *et al.* (1955) found none by the differential-hydrolysis method. This procedure, which measures the difference between total and alkali-labile phosphorus, is not very satisfactory when the proportion of alkali-resistant material is small. In addition to the value of 2·5 % found by Kaucher *et al.* (1943), Yanamoto (1954*a*) found 2·6 % of sphingomyelin in fertile and 7·7 % in 'wind' (infertile?) eggs. van Handel (1954) reported 4·5 and 5·5 % of sphingomyelin in egg phospholipids, but these higher values are not consistent with nitrogen/phosphorus ratios of 1·00 and 1·01 reported by this worker for the total phospholipids, since sphingomyelin contains two nitrogen atoms. Moreover, the alkali-resistant substance actually assayed was characterized only by a choline/phosphorus ratio of unity, a criterion insufficient to distinguish sphingomyelin from lecithin or from choline-containing plasmalogens. These figures, therefore, may be too high.

Egg phospholipids have been found to contain not more than traces of plasmalogen (Feulgen & Bersin, 1939). Thiele (1955) found less than 0·2 %, mostly in the lecithin fraction, but Yanamoto (1954*b*) reported 2·1 % in fertile and 1·0 % in 'wind' eggs.

Amino acid nitrogen was reported to be absent by Chargaff *et al.* (1942) and by van Handel (1954). Sulser (1954) separated seven ninhydrin-reacting substances from hydrolysates of egg phospholipid, and estimated the equivalent of 0·17 % of phosphatidylserine, but reported no measures to exclude adventitious amino acids.

Inositol was found in hydrolysates of egg phospholipids by Celmer (1949) and was also demonstrated qualitatively by Sulser (1954).

Phospholipid analyses so far described in the literature have been based upon the examination of water-soluble hydrolysis products from the crude materials, or from fractions obtained by counter-current distribution. Although valuable information as to the complexity of the mixture is gained by such methods, positive identification of the parent substances cannot usually be made on these data alone. Surprisingly little attention has been paid to the fatty portion of the molecule; it has, for example, been shown only recently (Hack, 1953; Rapport & Alonzo, 1955*a*) that the 'lecithin' of ox heart, prepared by precipitation with cadmium chloride, contains a major proportion of plasmalogen, although a determination of the fatty acid/phosphorus or ester/phosphorus ratio would have revealed the deficiency of carboxylic ester.

The present investigation arose from the observation that crude egg phospholipid and lecithin prepared from it, either by chromatographic separation on alumina (Hanahan, Turner & Jayko, 1951) or by the cadmium chloride procedure (Pangborn, 1951), contained small amounts of lyso compounds, the presence of which could be demonstrated by chromatography on silicic acid (Lea, Rhodes & Stoll, 1955). By the use of this method and of an improved alumina-column technique the main components of egg phospholipids have now been quantitatively separated, and the minor components concentrated sufficiently to permit their identification and estimation.

The distribution of the fatty acids in egg phosphatidylcholine and phosphatidylethanolamine has been considered in a recent communication (Rhodes & Lea, 1956*a*).

EXPERIMENTAL

Analytical methods. Total P and N, I_2 val. and fatty acids were determined as previously described (Lea *et al.* 1955); choline was determined by the method of Entenman, Taurog & Chaikoff (1944).

Carboxylic esters were determined by the method of Rapport & Alonzo (1955*b*) in samples too small to allow an accurate titration of fatty acids. A standard of pure methyl palmitate (Hormel Foundation) was included in all determinations.

Amino N in intact phospholipids was estimated by reaction with ninhydrin (Lea & Rhodes, 1955). Ethanolamine and amino acid N were determined, after hydrolysis,

by the Van Slyke manometric procedure and by the fluorodinitrobenzene (FDNB) method of Axelrod, Reichenthal & Brodie (1953). Reagent volumes were slightly modified in the latter method to avoid the necessity for pipetting the 6 and 8 ml. volumes required in the original procedure.

Inositol was determined after hydrolysis in 6N-HCl for 40 hr. by the method of Campling & Nixon (1954).

Lysolecithin and lysophosphatidylethanolamine were determined by the decrease in ester content (Shapiro, 1953) after incubation with lecithinase *B* of *Penicillium notatum* (Fairbairn, 1948). The reaction was complete within 1 hr. at 37° in acetate buffer, pH 4; no further change occurred up to 4 hr.

Sphingomyelin was estimated by the methods of Schmidt (1946) and of McKibbin & Taylor (1949). The sensitivity of the latter method was greatly increased by estimating the $CHCl_3$-soluble base hydrochloride, obtained after hydrolysis, by its colorimetric reaction with ninhydrin, in place of the total N. The determination could not be made quantitative in the absence of a sufficiently pure specimen of sphingosine to provide a standard.

Plasmalogens were estimated by reaction of the aldehydes with *p*-nitrophenylhydrazine (Wittenberg, Korey & Swenson, 1956). The results were calculated on the basis of a molecular extinction of 23 500 given by these authors for the *p*-nitrophenylhydrazone of *n*-decaldehyde.

Unless otherwise stated, all results are expressed on a molar basis.

Chromatographic methods. Chromatography on silica-impregnated filter paper and on silicic acid columns was carried out as previously described (Lea *et al.* 1955; Rhodes & Lea, 1956*b*). The silicic acid (Mallinckrodt) used in the present work was more retentive than some of those previously described, and methanol–$CHCl_3$ (30:70, v/v) was found more satisfactory than the 20:80 (v/v) mixture previously specified.

Chromatography of phospholipids on alumina has been employed by Hanahan *et al.* (1951) for the preparation of ovolecithin, by adsorption of the kephalin fraction from ethanolic solution. In this method a large volume of 95% ethanol was required to elute the lecithin, and its recovery was not complete before aminophospholipids appeared; the adsorbed kephalins were not recovered (see also Thiele, 1955).

A rapid and quantitative elution of the lecithin of egg-yolk phospholipids, together with recovery of the kephalin fraction in good yield, has now been achieved by chromatography on alumina, with other solvent mixtures, as follows. Alumina (40 g.; Aluminum Co. of America) was repeatedly stirred with methanol–$CHCl_3$ (1:1, v/v) and the supernatant liquid decanted until all very fine material had been removed; the slurry was then poured on to a glass-wool plug in a 1·5 cm. diam. tube. Egg phospholipid (2 g.) was applied in 20 ml. of the same solvent and elution commenced, after the walls of the tube had been washed down. The lecithin appeared in a concentrated band, free from amino N, and was recovered quantitatively in 150 ml. of eluate. The retained phospholipid was then eluted with ethanol–$CHCl_3$–water (5:2:2, by vol.), recoveries of P in this fraction corresponding to 92–98% of the kephalin applied. The rate of flow of this column was 10 ml./min. for the first solvent and 2 ml./min. for the second.

Pure phosphatidylcholine and phosphatidylethanolamine, prepared from egg, were recovered unchanged in quantitative yield when subjected to this procedure, indicating that no losses were occurring through hydrolysis on the column.

Preparation of egg phospholipids

Extraction from the yolk. Fertile eggs, obtained from the same hens and less than 24 hr. old, were used throughout the work. The whites of forty-eight eggs were separated and the yolks freeze-dried. After thorough mixing in a blender the dried material was packed *in vacuo* and stored at −30°.

Extraction of the dried yolk was carried out according to the scheme in Table 1, with solvents de-aerated by bubbling with CO_2. The operations shown in the left-hand column form a sequence typical of those generally employed in the extraction of phospholipids. The efficiency of these steps was tested by recovery of the mother liquors and residues, as shown in the right-hand column. The proportions of the total phospholipid recovered in the various fractions are shown in Table 2.

Only a small part of the total lipid P (4·1%) was present in the acetone extract AS 1, which contained the great bulk of the triglyceride fat, steroids and pigments. After removal of the solvent from this extract the lipids were applied in ethanol-free $CHCl_3$ to a silicic acid column, and the glycerides washed out (Borgström, 1952). The small quantity of phospholipid present was separated with some difficulty from intense yellow, green and red pigment bands, which were eluted first with methanol–$CHCl_3$ (2:98, v/v), leaving the phospholipid to be recovered in methanol–$CHCl_3$–water (70:25:5, by vol.). On silica-impregnated paper this phospholipid showed only two spots reacting with phosphomolybdic acid, one of which also reacted with ninhydrin; from their R_F values they appeared to be lecithin and phosphatidylethanolamine.

Most of the phospholipid of the dried yolk (92·7%) was recovered in the cold methanol–$CHCl_3$ extract E 1, leaving only 3·1% to be removed by subsequent treatment with the boiling solvent (extract E 2). The phospholipid recovered by silicic acid column chromatography of E 2 gave on analysis: N/P, 0·99; choline N/total N, 0·59; ethanolamine N/total N, 0·42. Chromatography on silica-impregnated paper showed the presence of lecithin, phosphatidylethanolamine and lysolecithin.

At this stage extraction of the lipids from the dried yolk was virtually complete, only traces of fatty acids being recoverable after saponification, acidification and extraction (E 3).

Purification by acetone. The cold methanol–$CHCl_3$ extract, E 1, containing most of the phospholipid, was purified by precipitation four times at 0° from a 15% (w/v) solution in light petroleum (40–60°), by the addition of 8 vol. of acetone. During this process a total of 14% of the original P passed into the acetone-soluble mother liquors (AS 2), corresponding to an average loss of 3·7% of the lipid P at each precipitation. Borgström (1952) has shown that this loss can be greatly reduced by the addition of $MgCl_2$ to the acetone, but this procedure requires the use of larger volumes of solvent and a molar $MgCl_2$/lipid P ratio of 2:1 which, it was considered, might lead to undesirable contamination of the phospholipid.

The mother liquors from the acetone precipitations (AS 2) contained 530 mg. of neutral fat, of I_2 val. 48, which corresponded to 6·4% by wt. of the extract E 1, and represented only 3% of the total neutral lipid recovered in AS 1 and E 1.

Table 1. *Extraction of the phospholipids from egg yolk*

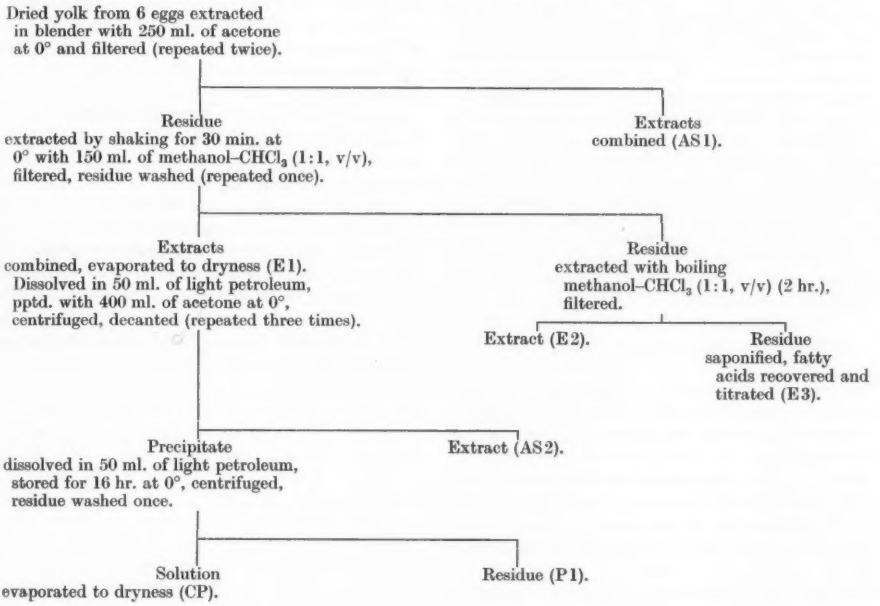
Dried yolk from 6 eggs extracted in blender with 250 ml. of acetone at 0° and filtered (repeated twice).
Residue extracted by shaking for 30 min. at 0° with 150 ml. of methanol–$CHCl_3$ (1:1, v/v), filtered, residue washed (repeated once).
Extracts combined (AS1).
Extracts combined, evaporated to dryness (E1). Dissolved in 50 ml. of light petroleum, pptd. with 400 ml. of acetone at 0°, centrifuged, decanted (repeated three times).
Residue extracted with boiling methanol–$CHCl_3$ (1:1, v/v) (2 hr.), filtered.
Extract (E2).
Residue saponified, fatty acids recovered and titrated (E3).
Precipitate dissolved in 50 ml. of light petroleum, stored for 16 hr. at 0°, centrifuged, residue washed once.
Extract (AS2).
Solution evaporated to dryness (CP).
Residue (P1).

Table 2. *Recovery of lipid P in main extracts*

Fraction	Lipid P in extract (as % of total P recovered)
Acetone extract of dried yolk (AS1)	4·1
Methanol–$CHCl_3$ extract (at 0°) (E1)	92·7
Methanol–$CHCl_3$ extract (boiling solvent) (E2)	3·1
Fatty acids recovered from residue (calculated as P) (E3)	0·2

Extraction of the dried yolk with acetone had therefore removed over 97% by wt. of the neutral lipids originally present.

Fraction insoluble in light petroleum. Of the total lipid P 2·1% was found in material insoluble in light petroleum at 0°, a procedure designed to take out sphingomyelin and fully saturated lecithins. This fraction contained considerable quantities of salts and amino acids (N/P, 1·48) which were completely removed, without loss of P, by treatment with a cellulose column (Lea & Rhodes, 1953).

Analysis of the purified material gave: N/P, 1·26; sphingomyelin/P, 0·26 (Schmidt, 1946); I_2 val., double bonds/P, 1·88.

Separation on an alumina column yielded 83% of choline-containing phospholipids and 15% of 'kephalins'. On impregnated paper the choline-containing phospholipids showed three phosphomolybdic acid-reacting spots corresponding to lecithin, sphingomyelin and lysolecithin; the 'kephalins' showed one ninhydrin-reacting spot corresponding to phosphatidylethanolamine.

After selective alkaline hydrolysis (Schmidt, 1946) of the whole fraction insoluble in light petroleum only the sphingomyelin spot remained: the recovery of a ninhydrin-reacting, $CHCl_3$-soluble base from this alkali-resistant material, after hydrolysis according to McKibbin & Taylor (1949), confirmed its probable identity with sphingomyelin.

The I_2 val. of the fraction insoluble in light petroleum, when corrected on the assumption that the sphingomyelin present contained one double bond, was only slightly below the average for the whole phospholipid, indicating that no doubly saturated glycerophospholipids had been selectively precipitated.

The fraction insoluble in light petroleum, therefore, consisted mainly of glycerophospholipids of average constitution, and, as shown below, the sphingomyelin present represented only a small proportion of the sphingomyelin of the whole phospholipid.

Conclusion. It can be concluded from these experiments that a procedure such as that usually employed for the preparation of egg phospholipids yields about 80% of the total as acetone-insoluble material. By precipitation with 5 in place of the 8 vol. of acetone used above, this yield could be increased to 87%, but the loss was still too great in a preparation to be used for analysis. On the basis of this work the following procedure was adopted for the preparation of material reasonably representative of the total phospholipids of the yolk.

Preparation of the total phospholipid

The dried yolk was treated with acetone, as in Table 1, and the small proportion of the phospholipids extracted with the neutral fat (AS1) was recovered on a silicic acid column as described above. This was added to the main bulk of the phospholipid obtained by extraction with methanol–$CHCl_3$ at 0° (E1). Purification of the combined crude phospholipid fractions with acetone was omitted, leaving the small amount of neutral fat known to be present to be removed in the first runnings from the chromatographic columns. As no well-defined fractionation had been achieved by light petroleum this step also was omitted.

Material prepared in this way contained 97% of the lipid P of the yolk; the remaining 3%, obtainable only by prolonged hot extraction, was not recovered because of the possibility of decomposition.

RESULTS

Removal of neutral fat. The triglyceride fat in the preparations of total phospholipid was not retained by either adsorbent in the solvent systems used. It was eluted from the alumina columns with the 'lecithin' fractions and, when this was subsequently applied to a silicic acid column, the neutral fat and pigment appeared with the solvent front, about 10 hold-up volumes ahead of the first phospholipid band.

Separation on alumina. The progress of a typical separation on alumina of a total egg-phospholipid preparation is shown in Table 3. The ninhydrin reaction of the fraction GL corresponded to the presence of less than 0·1% of phosphatidylethanolamine, and no trace of choline-containing phospholipid was found in the three fractions combined as GK. The constant value of the ester reaction of the three 'kephalin' fractions, recorded in the last column, shows that no liberation of fatty acids was occurring through hydrolysis on the column even in the presence of an aqueous eluent; these fractions had been adsorbed for periods of 1·2, 6 and 20 hr. respectively.

A number of estimations of the choline-containing phospholipids of egg-yolk preparations made by this method all gave values between 78 and 82%. No inositol was found in a hydrolysate of GL.

Separation on silicic acid. When chromatographed on silica-impregnated paper both fractions from the alumina column showed evidence of slower-moving phospholipids corresponding to lyso compounds; silicic acid columns were, therefore, used to separate these components. When run in methanol–chloroform (30:70, v/v) the diacylglycerophospholipids were recovered in the first peaks (Fig. 1).

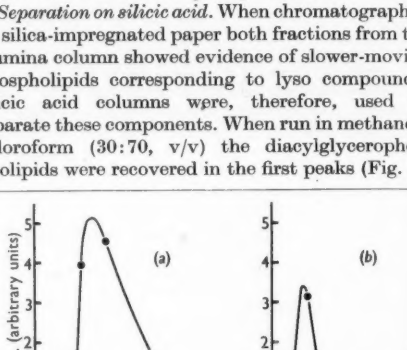

Fig. 1. Recovery of diacylglycerophospholipids by chromatography on silicic acid in methanol–$CHCl_3$ (30:70, v/v). (*a*) Fraction GL (98 mg. of lipid P applied to 120 g. column). (*b*) Fraction GK (17·2 mg. of lipid P applied to 30 g. column).

Table 3. *Separation of the total egg phospholipid on alumina*

P applied: 152·5 mg.; adsorbent: 160 g.

Fractions obtained	Solvent	Eluent (ml.)	P in fraction (as % applied)	Ester value*
Crude 'lecithin' (GL)	Methanol–$CHCl_3$ (1:1, v/v)	500	82·0	—
		10	0·02	—
Crude 'kephalin' (GK)	Ethanol–$CHCl_3$–water (5:2:2, by vol.)	500	14·4	11·09
		400	2·1	11·01
		400	1·2	11·30
		Total recovery	99·7	

* Colour yield (Rapport & Alonzo, 1955*b*)/mg. of P.

Table 4. *Chromatography of fractions GL and GK on silicic acid*

Material applied to column	Solvent	Fraction code	P recovered (as % applied)
GL	Methanol–$CHCl_3$ (30:70, v/v)	GL1	86·1
	Methanol–$CHCl_3$–water (70:25:5, by vol.)	GL2	12·8
		Total	98·9
GK	Methanol–$CHCl_3$ (30:70, v/v)	GK1	86·7
	Methanol–$CHCl_3$–water (70:25:5, by vol.)	GK2	13·3
		Total	100·0

When the P concentration in the effluent had fallen nearly to zero the solvent was changed to methanol–chloroform–water (70:25:5, by vol.) to expedite the removal of the more strongly held substances. This solvent, containing a small amount of water, was found to be more effective for the rapid and complete removal of phospholipids from silicic acid than the methanol–chloroform (75:25, v/v) or methanol alone previously used (Rhodes & Lea, 1956*b*). The fractionation of the lipid P is shown in Table 4.

Analysis of the 'lecithin' fractions GL1 and GL2. Fraction GL1 was chromatographically homogeneous and on analysis showed: N/P, 1·01; amino N/P, 0·001; fatty acids/P, 2·01; as required for phosphatidylcholine. It was not attacked by phospholipase *B*.

On silica-impregnated paper, fraction GL2 showed three spots (R_F values 0·5, 0·35, 0·25) reacting with phosphomolybdic acid, corresponding to phosphatidylcholine, sphingomyelin and lysophosphatidylcholine respectively.

Sphingomyelin was estimated in this fraction by the method of Schmidt (1946). Since no water-soluble ninhydrin-reacting substances were present, the N/P ratio in excess of unity provided a further estimate of the quantity of diaminophospholipid. The presence of a ninhydrin-reacting, chloroform-soluble base after hydrolysis (McKibbin & Taylor, 1949), in approximately the correct quantity, confirmed the presence of sphingosine.

Lysolecithin was demonstrated unambiguously and estimated by reaction with phospholipase *B*.

Analysed in this way fraction GL2 gave: N/P, 1·24; esters/P, 0·79; esters hydrolysed by phospholipase *B*/total esters, 0·70; sphingomyelin (Schmidt, 1946)/P, 0·24. From the ester values the content of lysolecithin in the fraction was 55 % and of lecithin 12 % which, with the 24 % of sphingomyelin, accounts for only 91 % of the P. The discrepancy is most likely due to inaccuracies inherent in the ester methods, and the remaining 9 % (less than 1 % of the total P) has been included as lecithin; the lysolecithin content given may therefore be slightly low. The fraction consisted, therefore, of sphingomyelin 24 %, lysolecithin 55 % and (by difference) lecithin 21 %. Confirmation of the composition of the fraction as a mixture of sphingomyelin with two glycerophospholipids was obtained by submitting the whole to selective alkaline hydrolysis (Schmidt, 1946) and rechromatographing the recovered phospholipid; only the sphingomyelin spot remained.

Fraction GL1, re-run on a silicic acid column, was recovered in a single peak in 30 % (v/v) methanol–chloroform, indicating that the lyso compound found in GL2 had not been produced by hydrolysis on the column.

Analysis of the 'kephalin' fractions GK1 and GK2. Analysis of fraction GK1 gave: N/P, 1·00; amino N/P, 1·00; esters/P, 1·99; inositol/P, 0·020. It was not attacked by phospholipase *B*; the fraction was, therefore, nearly pure phosphatidylethanolamine.

Fraction GK2, as eluted from the silicic acid column, still contained amino acid contaminants. These were removed on a cellulose column (Lea & Rhodes, 1953) without loss of lipid P: the N/P ratio of the purified material was then 1·01. On silica-impregnated paper this material showed only one spot at R_F 0·58 reacting with ninhydrin or phosphomolybdic acid, well separated from a phosphatidylethanolamine marker at R_F 0·85. Reaction with phospholipase *B* confirmed the presence of lysophosphatidylethanolamine and estimated its quantity. Inositol was estimated among the hydrolysis products.

Analysed in this way fraction GK2 gave: N/P, 1·01; esters/P, 1·26; esters hydrolysed by phospholipase *B*/total esters, 0·69; inositol/P, 0·125.

From these data lysophosphatidylethanolamine accounted for 87 % of the lipid P of the fraction; the remaining 13 % was in equimolar ratio to the inositol content (12·5 %). This result was confirmed by analysis of the corresponding fraction of another phospholipid preparation (H), which gave lysophosphatidylethanolamine 83 %, inositol/P, 0·20. In the absence of other phospholipids in these fractions, the evidence suggests that the inositide of egg contains ethanolamine, inositol and phosphorus in equimolar proportions. It may be further calculated that the ratio of fatty acid esters to P in this compound was approximately 3:1 in both experiments, but the significance of this figure is doubtful in view of errors inherent in the ester determination.

Although in the analysis reported fraction GK2 contained only part of the inositide, with preparation H, when the solvent was changed earlier, the entire inositide was recovered in the second fraction (HK2).

Amino acid N. The 'kephalin' fractions GK1 and GK2 were treated on cellulose columns to remove water-soluble contaminants, and the bases obtained on acid hydrolysis were then treated with fluorodinitrobenzene (FDNB) (Axelrod *et al.* 1953). Of the derivatives obtained from the phosphatidylethanolamine fraction GK1 1·2 % were acidic, and paper chromatography showed the presence of serine; a second spot may have been threonine. No amino acids were found in fraction GK2. The amino acid content of the total phospholipid was, therefore, 0·18 %.

Plasmalogen. The plasmalogen contents of the total phospholipid prepared from three batches of eggs were estimated. In view of the danger of obtaining high results owing to interference by

Table 5. *Composition of the total phospholipid of egg yolk derived from analysis of the chromatographically separated fractions*

Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; In, inositide. —, Absent chromatographically.

	P (as % of initial)						
Fraction	PC	PE	lyso PC	lyso PE	SM	In	Total
GL1	70·6	<0·1	0·0	0·0	—	0·0	70·6
GL2	2·2*	0·0	5·8	0·0	2·5	0·0	10·5
GK1	—	15·0	0·0	0·0	—	0·3	15·3
GK2	—	—	—	2·1	—	0·3	2·4
Total	72·8	15·0‡	5·8	2·1	2·5	0·6	98·8†

* Estimated by difference.
‡ Contains 0·2% of phosphatidylamino acids.
† Contains 0·9% of plasmalogen.

aldehydic degradation products from autoxidized fatty acids, and of the destruction of plasmalogens by hydrogenation fractions from the columns were not examined individually. Plasmalogen contents of 0·9, 1·2 and 1·6% were found.

The results of the analyses of the several fractions, calculated on the basis of the original lipid P, are presented in Table 5. The last line of the table gives the overall composition of the total phospholipid.

DISCUSSION

In so far as comparison is possible, the results obtained in the present work by the application of chromatographic methods to the analysis of egg-yolk phospholipids agree fairly well with the most recent of the published data based on the examination of hydrolysis products. The proportion of choline-containing phospholipids found was close to 80%, including 2·5% of sphingomyelin. The amino acid nitrogen was less than 0·2%. The plasmalogen content, as measured by the most recently developed method, was less than 1%, and was concentrated mainly in the lecithin fraction.

Chromatography on silicic acid has permitted further fractionation of both the 'lecithin' and the 'kephalin' fractions from alumina columns, with the recovery in each case of lyso compounds, the presence of which had previously been reported (Lea *et al.* 1955; Rhodes & Lea, 1956*b*). In the earlier work, lysophosphatidylcholine had been obtained by direct chromatography of the crude phospholipids on silicic acid, and the presence of lysophosphatidylethanolamine inferred from the weak ninhydrin reaction of the phosphatidylcholine fraction obtained from the same columns, as well as from the chromatographic behaviour of artificial phospholipid mixtures.

The methods now used have concentrated the small amount of lysophosphatidylethanolamine present into one fraction, of which it comprised over 80%, thus allowing its positive identification and estimation. The inositide of the yolk phospholipid could be concentrated, as the only other major constituent, in the same fraction.

The analysis reported in this paper was carried out on eggs obtained from one flock at one time. In view of the range of fatty acid/phosphorus ratios observed for a number of egg-phospholipid preparations in previous work (Lea *et al.* 1955), it seems likely that some variation, particularly in the proportion of lyso compounds present, may occur. The fatty acid/phosphorus ratio of the total phospholipid now analysed, as calculated from the data in Table 5, was 1·87, a figure within the range previously reported.

The improved solvent system employed with alumina permits the rapid and complete separation of the phospholipids into choline-containing and non-choline-containing fractions, and provides a more rapid and convenient procedure for the preparation of the crude 'lecithin' of egg yolk than that originally described by Hanahan *et al.* (1951). It must, however, be followed by a treatment on a silicic acid column to remove lysolecithin and sphingomyelin if pure phosphatidylcholine is required. The separation of the lyso compounds obtained from egg yolk by treatment with snake venom is also more conveniently carried out on the alumina column than by the silicic acid column previously described (Lea *et al.* 1955).

Application of the improved alumina-column technique to tissue lipids rich in phosphatidylserine and inositol lipids might well prove less successful than in the present case, owing to the low solubility of these phospholipids in the ethanol-rich eluent used.

Phosphatidylethanolamine is still obtained most easily by direct chromatography of the acetone-insoluble phospholipids of egg yolk on silicic acid (Lea *et al.* 1955; Lea, 1956).

SUMMARY

1. The extraction and purification of hen's egg phospholipids have been examined, and a mild procedure yielding 97% of the total material has been devised.

2. The crude phospholipid was separated chromatographically on alumina into choline-containing and non-choline-containing fractions, with quantitative recovery of both.

3. By further separation on silicic acid the two major constituents were recovered in a practically pure condition, and the minor constituents concentrated sufficiently to permit identification and estimation.

4. The egg phospholipid examined contained phosphatidylcholine 73·0, lysophosphatidylcholine 5·8, sphingomyelin 2·5, phosphatidylethanolamine 15·0, lysophosphatidylethanolamine 2·1 and inositol phospholipid 0·6, all as moles %.

5. The plasmalogen content of the whole phospholipid was 0·9%, most of which was present in the lecithin fraction. The phosphatidylethanolamine fraction contained a small proportion (0·2% of the total phospholipid) of amino acid-containing phospholipid.

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Some Effects of Propionate upon Acetate Metabolism in Animal Tissue Slices

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A number of instances of inhibitory effects of propionic acid on metabolic reactions have been reported. Grafflin & Green (1948) found that the oxidation of acetate by washed rabbit-kidney particles ('cyclophorase') was inhibited by propionate, which itself was not oxidized. The oxygen uptake of sheep-rumen epithelial tissue was shown (Pennington, 1954) to be markedly depressed by propionate in a medium without bicarbonate, but was increased by propionate in a medium buffered with bicarbonate–carbon dioxide. It was suggested that, in the absence of carbon dioxide, the metabolism of propionyl-coenzyme A was blocked and that coenzyme A, which would normally be available for the metabolism of endogenous substances, was immobilized as propionyl-coenzyme A. Propionate suppresses ketone-body formation by rumen epithelium from pyruvate and decreases slightly the uptake of pyruvate (Pennington & Sutherland, 1956*a*). Lang & Bässler (1953) found that propionate inhibited the oxygen uptake of particle preparations from rabbit liver and kidney in the presence of low levels of succinate; the formation of acetoacetate from acetate by the liver preparations was depressed also. These effects were attributed to effective competition by propionate for coenzyme A.

It has been established that propionate inhibits the growth of many micro-organisms (see Heseltine, 1952). Hill (1952) showed that the suppression of growth of *Streptococcus faecalis* by propionate could be reversed by acetate. He suggested that, by combining with coenzyme A, propionate interferes with the oxidative decarboxylation of pyruvate to acetate.

The ruminant liver normally metabolizes considerable quantities of propionic acid, formed in the rumen. Consequently, any inhibitory effects of propionate may be of considerable physiological importance to this class of animals. The present studies were concerned with possible effects of propionate on the metabolism of acetate, which is present in relatively high concentration in the blood of ruminants. Most of the experiments described were carried out, for convenience, with rat liver. It subsequently became evident, however, that there was a marked species difference in the magnitude of the effects of propionate.

EXPERIMENTAL

Labelled compounds. These were obtained from the Radiochemical Centre, Amersham. (The D-[^{14}C]glucose is prepared by tobacco-leaf photosynthesis and is expected to be uniformly labelled.)

Animals. The rats were Hooded Lister strain, 3–4 months old, and were given rat cubes *ad lib.*; males were used in every case except Expt. 3, Table 7. The sheep livers were obtained from Scottish Blackface ewes, which had been fed on adequate amounts of hay and concentrates.

Tissue slices. The slices were prepared either with the tissue-chopping machine of McIlwain & Buddle (1953) or by free-hand slicing with the technique of Deutsch (1936). Considerable differences, summarized in Table 1, were observed between the rates of respiration, of ketone-body formation and of $^{14}CO_2$ formation from [*carboxy*-^{14}C]acetate

Table 1. *Effect of slicing technique on metabolism of rat-liver slices*

Liver slices (500 mg., wet wt.) were incubated for 3 hr. at 38° in 3 ml. of Ringer–phosphate solution containing [*carboxy*-^{14}C]acetate (0·01 M). The gas phase was O_2. $Q_{O_2} = \mu$l. of O_2/mg. dry wt./hr.

Expt. no.	Method of slicing	Q_{O_2} Initial	Q_{O_2} After 3 hr.	Ketone bodies produced (μmoles/100 mg. dry wt.)	Acetate carboxyl-C in CO_2 (μatoms/100 mg. dry wt. of tissue)
1	Hand	8·19	4·86	12·46	8·36
	Machine, 0·416 mm.*	7·27	2·54	4·64	2·95
2	Hand	7·06	5·21	—	10·28
	Machine, 0·416 mm.*	7·49	1·62	—	5·95
	Machine, 0·572 mm.*	7·02	1·71	—	4·46
	Machine, 0·728 mm.*	7·07	2·46	—	6·41

* The machine was set to cut slices of the stated thickness.

by rat-liver slices cut by the different procedures. It is evident that the respiratory activity of machine-cut slices declined more rapidly than that of slices cut by hand. The method of slicing used in each experiment is stated in the legends to the tables.

The slices were kept in ice-cold Ringer solution before use. The time from killing to the end of the equilibration period was 60–90 min. All dry weights given are final dry weights. The metabolic data presented are, with few exceptions, means obtained from two runs with separate lots of tissue. Pooled slices, usually from a single animal, were used in any one numbered experiment.

Medium. The basal medium was prepared by mixing 90 parts of Krebs–Ringer solution (Cohen, 1949) from which $CaCl_2$ was omitted, 10 parts of 0·1 M-Na phosphate buffer (pH 7·2) and 7·5 parts of $NaHCO_3$ (0·154 M). Fatty acids and pyruvic acid were added as sodium salts. The flasks were gassed with $O_2 + CO_2$ (95:5, v/v) and the final pH was 7·1. For use with sheep tissue the concentration of all the components of the Ringer solution was increased to give a total concentration of 0·167 M. For the respiration measurements of Table 1, the bicarbonate was omitted and the Warburg flasks were gassed with O_2.

General procedure and measurement of $^{14}CO_2$. The medium and slices were shaken (80–90 strokes/min.) at 38°, either in Warburg vessels or in specially constructed flasks. The latter were similar to those described by Pennington & Sutherland (1956*b*), with the addition of a centre well like that of Warburg flasks. At the end of the experiment 0·5 ml. of N-HCl was injected to kill the tissue and liberate bound CO_2, then 1 ml. of 2 N-NaOH (CO_2-free) was injected through the rubber cap into the centre well and the flasks were shaken for 75–90 min. to allow absorption of the labelled CO_2. The $^{14}CO_2$ was counted as $Ba^{14}CO_3$.

When smaller quantities of medium and tissue were required, Warburg flasks were adapted for use by replacing the stopper by a rubber cap. To allow gassing of the flasks a hypodermic needle was inserted temporarily through the cap. (If such a cap is not readily available it can be made easily by boring a wide hole in a rubber stopper to within a short distance of the base. The stopper is inserted and the top turned down over the outside of the glass.) At the end of the experiment HCl was injected into the side arm and tipped into the main compartment. NaOH was then injected into the side arm to absorb the $^{14}CO_2$.

Ketone bodies. The tissue was washed three times with warm water and the medium and washings were combined. After a portion of the liquid had been neutralized, protein was precipitated with solutions of $ZnSO_4$ and $Ba(OH)_2$. The filtrate was used for determination of acetone by the method of Thin & Robertson (1952) and of total ketone bodies and β-hydroxybutyric acid by the method of Greenberg & Lester (1944) as modified previously (Pennington & Sutherland, 1956*a*). Acetoacetate was determined by difference.

For radioactivity measurements on ketone bodies, these were converted into acetone by refluxing the protein-free filtrates with chromic acid, as in the procedure of Greenberg & Lester (1944). The acetone was aerated into a saturated solution of 2:4-dinitrophenylhydrazine in 2 N-HCl (5 ml.) and the acetone dinitrophenylhydrazone extracted with CCl_4 (2 ml.). After colorimetric measurement of the dinitrophenylhydrazone, the CCl_4 was removed by evaporation and the radioactivity of the dinitrophenylhydrazone was measured by direct counting.

In one experiment, after a portion of the filtrate had been refluxed with chromic acid, the acetone was distilled off and precipitated, as the Hg complex, with Denigè's reagent. The complex was decomposed with 10% HCl, the acetone distilled off and the distillate aerated as described above. The specific activity of the dinitrophenylhydrazone was 88% of the value obtained by the usual procedure.

The radioactivity in the carboxyl group of acetoacetic acid or β-hydroxybutyric acid was not measured. In two instances (Table 7, Expt. 1; Table 8, Expt. 1) only the acetone and acetoacetic acid were assayed for radioactivity; instead of the samples being refluxed with chromic acid they were treated with aniline citrate (Edson, 1935), followed by aeration etc.

Fatty acid uptake. The disappearance of labelled fatty acid was determined by steam-distilling 1 ml. of the medium (and washings) in a small Markham still, after precipitation of protein with metaphosphoric acid. The distillate was made slightly alkaline, evaporated to a small volume and portions were evaporated to dryness on nickel planchets and counted.

Radioactivity in lipids. The washed tissue was extracted five times by refluxing with 10 ml. of ethanol–ether (3:1, v/v) for 5 min. The combined extracts were evaporated to dryness, 5 ml. of hexane and 2 ml. of water were added and the tube was shaken vigorously for 15 min. (Katz & Chaikoff, 1954). Portions of the hexane layer were plated and counted. There was no serious interference from radioactive acetate in this procedure.

RESULTS

The effect of propionate on the formation of $^{14}CO_2$ from [*carboxy*-^{14}C]acetate by rat-liver slices is shown in Table 2, which also shows the effect of acetate upon the oxidation of labelled propionate. It will be seen that the oxidation of acetate, as measured by the appearance of isotope in the CO_2, was almost completely suppressed by propionate. On the other hand, the oxidation of propionate was actually increased by acetate in each instance.

In another experiment, not recorded, there was no difference in the extent of inhibition of acetate oxidation produced by commercial sodium propionate which had been purified by recrystallizing three times from aqueous ethanol and then steam-distilling as propionic acid.

Table 3 shows the effect of lower concentrations of propionate upon acetate oxidation. An inhibitory effect is noticeable, even when the concentration of propionate was only one-twentieth that of the acetate. The 1 hr. incubation was carried out because of the likelihood that such small amounts of propionate would be completely used up within 3 hr. However, by comparison of the 1 and 3 hr. results, it is evident that there was very little oxidation of acetate by machine-cut slices after the first hour. This was confirmed by another experiment, in which the labelled acetate was added after the tissue had been incubated in the basal medium for varying periods. It appears, therefore, that the

Table 2. *Formation of labelled $^{14}CO_2$ from acetate and propionate by rat-liver slices*

The slices (1 g. and 500 mg. respectively) in Expts. 1 and 2 were cut by chopper (set at 0·416 mm.); 600 mg. of slices, cut by hand, were used in Expts. 3 and 4. All results are corrected to 100 mg. (final dry wt.). The flasks contained Ringer–phosphate–bicarbonate (10 ml. in Expt. 1, 3 ml. in Expts. 2–4) and the additions shown. They were incubated at 38° for 3 hr. (Expts. 1 and 2), 30 min. (Expt. 3) or 2 hr. (Expt. 4).

Expt. no.	Additions: Labelled [*carboxy*-^{14}C]	Additions: Unlabelled	Labelled C in CO_2 (μatoms)
1	Acetate (0·01 M)	None	4·60
	Acetate (0·01 M)	Propionate (0·01 M)	0·21
2	Acetate (0·01 M)	None	4·45
	Acetate (0·01 M)	Propionate (0·01 M)	0·08
	Propionate (0·01 M)	None	1·32
	Propionate (0·01 M)	Acetate (0·01 M)	2·22
3	Acetate (0·01 M)	None	2·49
	Acetate (0·01 M)	Propionate (0·01 M)	0·06
4	Propionate (0·01 M)	None	6·96
	Propionate (0·01 M)	Acetate (0·01 M)	8·77
	Propionate (0·005 M)	None	6·94
	Propionate (0·005 M)	Acetate (0·01 M)	8·01
	Propionate (0·0025 M)	None	4·55
	Propionate (0·0025 M)	Acetate (0·01 M)	6·00

Table 3. *Effect of various concentrations of propionate on acetate oxidation by rat-liver slices*

Slices (250 mg., wet wt.) cut by the chopper set at 0·416 mm. were used in 3 ml. of medium. [*carboxy*-^{14}C]-Acetate (0·01 M) was present in all the flasks, which were incubated at 38°. The figures are calculated to 100 mg. (dry wt.) of tissue.

Expt. no.	Concn. of propionate (M)	Incubation period (hr.)	Acetate carboxyl-C in CO_2 (μatoms)
1	0	3	4·16
	0·01	3	0·11
	0·005	3	0·15
	0·0025	3	0·20
2	0	3	4·32
	0·01	3	0·11
	0·00125	3	1·64
	0·0005	3	3·12
	0	1	4·12
	0·0005	1	2·00

Table 4. *Oxidation of acetate and propionate by slices of various animal tissues*

Slices cut by the chopper set at 0·416 mm. were incubated in 3 ml. of medium at 38° for 3 hr. The figures are calculated to 100 mg. (dry wt.) for the rat tissues, and 300 mg. (wet wt.) of rumen epithelium was used.

Tissue	Additions (0·01 M): Labelled [*carboxy*-^{14}C]	Additions (0·01 M): Unlabelled	Labelled C in CO_2 (μatoms)
Kidney (rat)	Acetate	None	85·9
	Acetate	Propionate	30·9
	Propionate	None	53·3
	Propionate	Acetate	43·5
Heart (rat)	Acetate	None	0·79
	Acetate	Propionate	0·37
Diaphragm (rat)	Acetate	None	2·91
	Acetate	Propionate	1·15
Rumen epithelium (sheep)	Acetate	None	1·20
	Acetate	Propionate	0·51

ability of such slices to oxidize acetate declines even more rapidly than the Q_{O_2} (cf. Table 1).

The effect of propionate upon acetate oxidation by some other tissues was examined (Table 4). Although acetate oxidation was depressed in each case, the relative effect of propionate was much less than was observed with rat liver. With kidney tissue, in contrast with liver, acetate slightly decreased the conversion of the propionate carboxyl-C into CO_2.

Certain amino acids, namely methionine, isoleucine and valine, are believed to form propionic acid (or propionyl-coenzyme A) when metabolized by liver (Patwardhan, 1954; Coon, Abrahamsen & Greene, 1952; Kinnory & Greenberg, 1953). The effect of each of these on acetate oxidation was measured (Table 5). Butyrate and *iso*valerate, both of which may be expected to compete for available coenzyme A, were also examined. The latter, like propionate, inhibits the respiration of rumen epithelium in a bicarbonate-free medium (Annison & Pennington, 1954). The effects of valine and butyrate were particularly marked, although none of the compounds tested were as effective as propionate in suppressing acetate oxidation.

Edson (1936) showed that sorbitol is an extremely strong inhibitor of endogenous ketone-body production by liver slices from fasted rats, being in this respect more effective than glucose. Table 6 shows the effect of sorbitol and of glucose on the metabolism of labelled acetate. The effects of acetate and propionate on the oxidation of labelled glucose are included in this table. It may be seen that neither sorbitol nor glucose has any marked effects on the conversion of acetate into CO_2 or ketone bodies. Both increased somewhat the incorporation of the isotope into the lipid. The effect of propionate on glucose oxidation was not comparable with its effect on the oxidation of acetate.

More extensive studies of the effects of propionate on acetate metabolism were undertaken in further experiments (Table 7), in which acetate uptake and ketone-body formation were also measured. The strong inhibition of acetate oxidation by propionate was paralleled by its effect on the total amount of acetate metabolized and on the incorporation of the isotope into ketone bodies (excluding the carboxyl groups of acetoacetate and β-hydroxybutyrate which were not assayed). A point of interest in Table 7 is the relatively large proportion of ketone bodies accounted for as acetone.

Table 8 shows the results of similar experiments carried out with sheep liver. Comparison with Table 7 reveals considerable quantitative differences in the effect of propionate on acetate metabolism. Both the overall disappearance of acetate and the formation of $^{14}CO_2$ were influenced far less by propionate than in rat liver. There are two other

Table 5. *Effect of various other compounds on acetate oxidation by rat liver*

Slices cut by the chopper set at 0·416 mm. were incubated in 3 ml. of medium for 3 hr. at 38°. All flasks contained [*carboxy*-^{14}C]acetate (0·01 M). The amino acids were neutralized. The figures are calculated to 100 mg. (dry wt.) of tissue.

Expt. no.	Addition		Acetate carboxyl-C in CO_2 (μatoms)
1	None		3·36
	Propionate	(0·01 M)	0·11
	DL-Methionine	(0·02 M)	1·44
	DL-Isoleucine	(0·02 M)	2·12
	DL Valine	(0·02 M)	0·63
2	None		2·85
	Propionate	(0·01 M)	0·09
	n-Butyrate	(0·01 M)	0·42
	*iso*Valerate	(0·01 M)	1·06

Table 6. *Mutual interaction of sorbitol, glucose and fatty acids, metabolized by rat-liver slices*

Slices cut by the chopper set at 0·416 mm. were used. In Expt. 1, 600 mg. of slices was incubated in 3 ml. of medium for 2 hr. at 38°. In Expt. 2, 1 g. of slices was incubated in 10 ml. of medium for 3 hr. The concn. of each substrate was 0·01 M. The quantities given are the totals for each experiment.

Expt. no.	Additions		^{14}C (μatoms) in		
	Labelled	Unlabelled	CO_2*	Total lipids	Ketone bodies (C_{2-4})
1	[*carboxy*-^{14}C]Acetate	None	4·43	0·31	0·81
	[*carboxy*-^{14}C]Acetate	Sorbitol	4·30	0·40	0·84
	[*carboxy*-^{14}C]Acetate	D-Glucose	4·61	0·39	0·78
2	[*carboxy*-^{14}C]Acetate	None	5·50	—	—
	[*carboxy*-^{14}C]Acetate	Sorbitol	5·40	—	—
	[*carboxy*-^{14}C]Acetate	D-Glucose	6·20	—	—
	D-[^{14}C]Glucose	None	3·71	—	—
	D-[^{14}C]Glucose	Acetate	2·64	—	—
	D-[^{14}C]Glucose	Propionate	3·33	—	—

* The glucose is assumed to be uniformly labelled.

marked differences between the results obtained with rat and sheep tissue. The total ketone-body production by sheep-liver slices was only a fraction of that observed with the rat tissue, and acetone formation by the sheep liver was negligible.

The fact that, in rat liver, acetate uptake and CO_2 and ketone-body production were all inhibited by propionate tends to suggest that the inhibition occurs at an early stage in the metabolism of acetate. In an attempt to define further the site of inhibition,

Table 7. *Effect of propionate on metabolism of acetate by rat-liver slices*

Slices (1 g., wet wt.) incubated at 38° for 3 hr. in 5 ml. of medium containing [*carboxy*-^{14}C]acetate (0·01 M). Propionate was added where indicated. The ketone-body production in control flasks without substrate is shown in parentheses. The slices in Expt. 1 were cut by chopper set at 0·416 mm., those in Expts. 2 and 3 by hand. The quantities given are the totals for each experiment.

Expt. no.	Propionate (0·01 M, where present)	Acetate used (μmoles)	Acetate carboxyl-C (μatoms) in CO_2	Acetate carboxyl-C (μatoms) in Ketone bodies (C_{2-4})	Ketone bodies formed (μmoles)* Total	Acetone	β-Hydroxybutyrate	Acetoacetate
1	–	23·4	14·50	1·50†	7·05 (4·95)	2·52 (2·05)	1·55 (0·20)	2·98 (2·70)
	+	2·4	0·31	0·03†	3·40	1·05	0·45	1·90
2	–	31·7	17·37	1·70	12·30 (11·17)	4·72 (5·14)	5·25 (4·57)	2·33 (1·46)
	+	0·9	0·77	0·07	5·03	1·13	2·55	1·35
3	–	21·9	15·48	1·19	7·54 (4·68)	3·55 (2·81)	2·63 (1·35)	1·36 (0·52)
	+	0·7	0·93	0·05	2·04	0·67	1·03	0·34

* By chemical analysis. † Acetoacetate + acetone, only.

Table 8. *Effect of propionate on metabolism of acetate by sheep-liver slices*

Conditions as for Table 7. The slices in Expt. 1 were cut by chopper set at 0·416 mm., those in Expts. 2 and 3 by hand. The quantities given are the totals for each experiment.

Expt. no.	Propionate (0·01 M, where present)	Acetate used (μmoles)	Acetate carboxyl-C (μatoms) in CO_2	Acetate carboxyl-C (μatoms) in Ketone bodies (C_{2-4})	Ketone bodies formed (μmoles)* Total	Acetone	β-Hydroxybutyrate	Acetoacetate
1	–	22·1	5·90	0·149†	1·76 (0·84)	0 (0)	1·11 (0·27)	0·65 (0·57)
	+	13·8	1·12	0·006†	0·67	0	0·21	0·46
2	–	24·5	13·02	0·72	2·21 (2·07)	0 (0)	1·84 (1·66)	0·37 (0·41)
	+	14·0	9·69	0·15	1·25	0	1·02	0·23
3	–	22·8	13·91	0·92	2·92 (2·66)	0·11 (0·17)	1·84 (1·73)	0·97 (0·76)
	+	13·8	9·10	0·05	1·30	0·20	0·83	0·27

* By chemical analysis. † Acetoacetate + acetone, only.

Table 9. *Effect of propionate on metabolism of butyrate and pyruvate by rat-liver slices*

Slices (1 g., wet wt.) cut by hand were incubated at 38° for 2 hr. in 5 ml. of medium containing the additions shown. The ketone-body production in control flasks without substrate is shown in parentheses. The quantities given are the totals for each experiment.

Expt. no.	Additions	Pyruvate used (μmoles)	^{14}C (μatoms) in CO_2	^{14}C (μatoms) in Ketone bodies (C_{2-4})	Ketone bodies formed (μmoles) Total	Acetone	β-Hydroxybutyrate	Acetoacetate
1	[*carboxy*-^{14}C]Butyrate (0·006 M)	—	8·78	0·97	15·00 (6·32)	6·08 (2·14)	5·55 (2·18)	3·37 (2·00)
	[*carboxy*-^{14}C]Butyrate (0·006 M) + propionate (0·006 M)	—	9·55	—	18·80	3·04	7·05	8·71
2	[*carboxy*-^{14}C]Butyrate (0·01 M)	—	7·48	1·30	11·51 (4·35)	5·18 (2·37)	3·38 (1·98)	2·95 (0)
	[*carboxy*-^{14}C]Butyrate (0·01 M) + propionate (0·01 M)	—	9·02	1·38	13·35	3·10	7·73	2·52
3	[2-^{14}C]Pyruvate (0·006 M)	24·8	8·93	0·42	5·14 (3·91)	1·49 (1·36)	2·85 (1·77)	0·80 (0·78)
	[2-^{14}C]Pyruvate (0·006 M) + propionate (0·006 M)	16·2	4·83	0·01	2·95	1·07	1·34	0·54

the effect of propionate on the metabolism of labelled butyrate and pyruvate was studied (Table 9). The production of $^{14}CO_2$ and the uptake of pyruvate were depressed by propionate, although the effects were much less than the effects upon acetate metabolism; on the other hand, ketone-body production from pyruvate was virtually abolished by propionate. Propionate had little or no effect on the metabolism of butyrate.

DISCUSSION

The main finding in the work described above was that propionate strongly suppressed the metabolism of acetate by rat-liver slices. Sufficient is known of the pathways of acetate metabolism in liver to make it possible to consider possible sites of inhibition. Available evidence indicates that, although the carbon of acetate has several alternative fates in liver, the initial step common to all is the formation of acetyl-coenzyme A. Since propionate inhibited both the uptake of acetate and its conversion into carbon dioxide and ketone bodies, it seems likely that it blocks the combination of acetate with coenzyme A. This hypothesis is supported by the observations (Table 9) that the oxidation of pyruvate was decreased much less, and that of butyrate not at all, by propionate. Both these compounds, as far as is known, can be oxidized by liver only after the formation from them of acetyl-coenzyme A. If the site of inhibition by propionate is subsequent to acetyl-coenzyme A formation, it would be expected that the oxidation of pyruvate and butyrate would be likewise prevented.

A more detailed investigation of the nature of the inhibition will necessitate the purification of the enzymes involved. Such data of this type as are available at present, however, throw little light on the problem. Hele (1954) purified from ox-heart muscle what appears to be a single enzyme which reversibly converts acetate and propionate into their coenzyme A derivatives. The Michaelis–Menten constant is greater with propionate ($5{\cdot}00 \times 10^{-3}$) than with acetate ($1{\cdot}42 \times 10^{-3}$). Straightforward competition between acetate and propionate for an enzyme with these characteristics obviously would not explain the effectiveness of propionate in inhibiting acetate metabolism. No enzyme which has been shown to form coenzyme A derivatives from both acetate and propionate has been purified from liver. The fatty acid-activating enzyme purified from ox liver (Mahler, Wakil & Bock, 1953) formed coenzyme A derivatives from fatty acids with 4–12 carbon atoms; it also had slight activity towards propionate but none towards acetate. The acetate-activating fraction from pigeon liver (Chou & Lipmann, 1952) was apparently not tested with propionate. Drysdale & Lardy (1953) found that extracts of an acetone-dried powder from rat-liver mitochondria activated butyrate and higher acids but failed to activate acetate.

Activation of acetate and propionate by a common enzyme having a much greater affinity for propionate is an obvious possibility to explain the observed inhibition. This seems unlikely, however, since individually acetate and propionate are metabolized at about the same rates by liver slices. On the other hand, separate acetic and propionic thiokinases [for nomenclature see *Biochem. J.* (1956), **64**, 782] may exist in liver and the acetic enzyme may be blocked by propionate. Any mechanism considered for the inhibition of acetate metabolism by propionate must take into account the fact that butyrate also markedly inhibited acetate oxidation (Table 5). In this connexion the hypothesis (Avigan, Quastel & Scholefield, 1955) that the reactions of acetyl-coenzyme A are inhibited by acyl-coenzyme A compounds is of interest.

The antiketogenic activity of propionate has often been looked on merely as a property possessed as a result of its glycogenic nature, thus posing no separate problem concerning the mechanism of the antiketogenic effect. However, Quastel & Wheatley (1933) suggested that propionate inhibits acetoacetate formation (in this case, from butyrate) by competition for enzymes. More recent work (Lang & Bässler, 1953; Pennington & Sutherland, 1956*b*) and the data in the present paper point to a similar conclusion. In this respect, however, Table 9 is of particular interest. The incorporation of the labelled carbon of the pyruvate into ketone bodies was depressed by propionate to a relatively much greater extent than was its conversion into carbon dioxide. Possibly propionate may inhibit ketone-body formation by another mechanism, in addition to blocking the formation of acetyl-coenzyme A.

The failure of sorbitol appreciably to influence the conversion of acetate into carbon dioxide or ketone bodies (Table 6) throws no light on its antiketogenic effect. The small increase in the isotope appearing in the lipids accords with the suggestion of Blakley (1952) that sorbitol may stimulate fat synthesis. However, glucose, which was a less effective antiketogenic agent than sorbitol (Edson, 1936), also increased the incorporation of ^{14}C into the lipids.

The marked difference in the effect of propionate on acetate metabolism by rat and sheep liver is being investigated further. It is pertinent, perhaps, to mention the differences between acetate metabolism in rat and sheep mammary tissue observed by Folley and his co-workers (e.g. Folley & French, 1950; Balmain, Folley & Glascock, 1952). The relatively large proportion of acetone in the ketone bodies produced by rat- compared with sheep-liver slices may be due to non-enzymic decarboxylation,

in the medium, of acetoacetate. This process is known to be catalysed by amino groups and it was found that during incubation considerably more protein escaped into the medium from rat-liver slices than from sheep-liver slices. On the other hand, this may represent another metabolic difference between rat and sheep liver. It should be noted that, since the incorporation of isotope into ketone bodies was always much less than into carbon dioxide, only a small proportion of the $^{14}CO_2$ produced during incubation of the rat-liver slices would be derived from the decarboxylation of acetoacetate.

SUMMARY

1. Propionate, in equimolar concentration, largely abolished the production of $^{14}CO_2$ from carboxyl-labelled acetate by rat-liver slices. Butyrate, *iso*valerate, valine, isoleucine and methionine inhibited acetate oxidation to varying degrees. The oxidation of propionate was not decreased by acetate.

2. Acetate oxidation by slices of other rat tissues was inhibited by propionate, but to a lesser degree than with rat-liver slices.

3. The uptake of acetate and the incorporation of its carboxyl-carbon into ketone bodies by rat-liver slices were also strongly inhibited by propionate.

4. The oxidation of carboxyl-labelled butyrate by rat liver was not inhibited by propionate; the oxidation of $C_{(2)}$-labelled pyruvate was affected to a much smaller extent than that of acetate.

5. The effects of propionate on acetate metabolism by sheep-liver slices were much smaller than those observed with rat liver.

6. The endogenous ketone-body production of sheep liver was less than that of rat liver.

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The Protein Composition and Nucleic Acid Content of the Rat Uterus in Different States

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This work arose out of our interest in the proteins of the contractile mechanism of the uterus. Csapo (1950*a*) has reported that more myosin and actomyosin were present in extracts of human, rabbit and rat uteri at the end of pregnancy than in extracts from the non-gravid uterus. In the rabbit some weeks after ovariectomy there was a marked fall in the amount of these proteins present per gram wet weight of uterus, and injection of oestrone into castrated animals led to a gradual rise towards the normal value (Csapo, 1950*b*). Csapo used exclusively the viscosimetric method worked out by Balenovic & Straub (1942; see also Szent-Györgyi, 1945) for estimation of the purified proteins, and it is possible that in tissue extracts interfering substances might be present. The value for the myosin–actomyosin

content of human striated muscle (44 mg./g. wet wt.) reported by him is low compared with the value found by thorough extraction of rabbit muscle with salt solution and precipitation of the myosin and actomyosin by dilution: 53% of the total protein (Hasselbach & Schneider, 1951), i.e. 82 mg./g. wet wt. (see Dische, 1926). Further, the myosin–actomyosin fraction of Csapo makes up only 7–14% of the total protein of the uterus. For these reasons it seemed of interest to attempt a fractionation of the proteins of rat uterus on the lines used by Robinson (1952*a*) for embryo muscle, and to follow the changes in all these fractions in different states of the uterus. The changes in the myosin–actomyosin fraction, precipitated by dilution of the extract, could thus be compared with the changes in the other fractions and set against a proper background. Three different states of the uterus were chosen: in mid-oestrus, at the last day of pregnancy and at 3 weeks after ovariectomy.

Since deoxyribonucleoprotein is extracted by salt solutions and precipitated by dilution under conditions similar to those used for actomyosin, it became necessary to investigate also the amounts of deoxyribonucleic acid (DNA) in the tissue and in the dilution precipitates; ribonucleic acid (RNA) was also estimated.

A preliminary account of this work has already been published (Needham & Cawkwell, 1955).

METHODS AND MATERIALS

Fractionation of the proteins. The method used was based on that of Robinson (1952*a*).

(i) Preparation of the extract. The tissue was extracted with 1·25 M-KCl containing 0·066 M potassium phosphate, pH 7·9, and 0·0015 M adenosine triphosphate (ATP). Since the uterus after ovariectomy is difficult to disintegrate, being fibrous and slippery, the conditions of extraction were conditioned by the need to ensure that the organ in this state was thoroughly extracted. The routine adopted was as follows. Immediately after the death of the animals by rapid bleeding, the uteri were dissected out and cooled to about 4°. In the cold room, the organs were slit open on cold glass plates and the epithelial layer and any uterine secretion were scraped off by means of a flexible plastic pen. The analyses thus refer to the endometrium and myometrium together. The uteri were then chopped; the weighed tissue was placed in a cold mortar and left to cool in air at −12 to −15°. After 3 hr. it was ground for 10 min. in the room at −12° with 10 vol. of the extracting fluid and one-tenth of its weight of acid-washed sand. The mortar was removed to 4° and all subsequent operations were carried out at this temperature. After thawing was complete (about 30 min.) the contents were centrifuged for 10 min. at 600 *g* on the International refrigerated centrifuge. The residue was ground twice more, each time with 5 vol. of extracting solution. Next day the combined supernatants were again centrifuged for 10 min. as before, and then for 1 hr. at 41000 *g* in the Spinco ultracentrifuge, model L, to remove cell particles which might be carried down with the dilution precipitate. All values given for *g* are average values.

(ii) Precipitation of the proteins insoluble at low ionic strength. A known volume of the supernatant was diluted 2·5 times by the gradual addition of water and the pH was brought to 7·0 with acetate buffer, pH 4·5, by means of the glass electrode. Dilution was continued by slowly adding 4 vol. of 19% (v/v) ethanol with constant stirring. The white precipitate quickly flocculated; it was centrifuged down after some hours and dissolved in 0·6 M-KCl; the solution was made up to a known volume.

(iii) Extraction of the residue with 0·1 M-NaOH. The residue was ground in the mortar with the alkali (5 ml./g. of tissue), and centrifuged. This procedure was repeated twice more, the third extraction being left to stand overnight. The combined supernatants were made up to a known volume.

(iv) The residue was treated with an equal vol. of 10% (w/v) trichloroacetic acid and centrifuged. It was washed twice with 5% (w/v) trichloroacetic acid, then warmed with 2 ml. of conc. H_2SO_4. After the protein had dissolved, the sand was centrifuged down and the supernatant transferred to a Kjeldahl flask. The sand was washed three times with dilute H_2SO_4 and the washings were added to the flask. Estimations showed that only about 1·5% of the residue N remained with the sand.

Nitrogen estimations. These were made by the method of Chibnall, Rees & Williams (1943), with a Markham (1942) distillation apparatus on known amounts of fractions (i) to (iii), after removal of non-protein N by three extractions with 5% trichloroacetic acid; in fraction (iv) known samples were taken after incineration. Total protein N was estimated in triplicate on 50–100 mg. samples; these were well stirred with 5% (w/v) trichloroacetic acid in the centrifuge tubes and allowed to stand overnight. After centrifuging, the residue was washed twice with the trichloroacetic acid. The recovery in the four fractions was 96–98% of the total.

Estimation of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). This was done by a modification of the method of Schmidt & Tannhauser (1945), with removal of phospholipids by means of ice-cold *n*-butanol (P. D. Mitchell & J. Moyle, personal communication; see Needham & Cawkwell, 1956). When nucleic acid estimations were to be made on the whole tissue, the chopped material was prepared as usual, then freeze-dried to constant weight and finely ground. Weighed amounts of the powder were suspended in water (about 50 mg./3 ml.) for extraction. The DNA P and RNA P were estimated by Allen's method (1940) or, if the quantities were below 20 μg., by a modification of the method of Weil-Malherbe & Green (1951; see Needham & Cawkwell, 1956). Since the method of extraction with butanol had not previously been used for animal tissues, a number of parallel analyses were made in which the method of Reichert (1944) with the modifications of Mitchell & Moyle (1951) was used for extraction of the phospholipids. Good agreement was found.

Estimation of collagen. This was estimated by means of the hydroxyproline content, by the method of Neuman & Logan (1949, 1950). Hydroxyproline (L. Light and Co. Ltd., Colnbrook, Bucks) was used for the standard; this gave the same standard curve as a specimen five times recrystallized by Dr G. R. Tristram. Occasional difficulties with fading of the colour to be measured were overcome by redistillation of the A.R. *iso*propyl alcohol used. Since the collagen content of the uterus is high, the small error due to tyrosine was

Table 1. *Distribution of protein N in the rat uterus in different states*

Results (mg./g. wet wt.) are expressed as means ± S.E.M. The experiments were done on groups of four to six uteri in oestrus, on single uteri in pregnancy and on groups of nine to twelve uteri after ovariectomy. The number of groups or of single animals used is shown in parentheses.

	Oestrus	End of pregnancy	After ovariectomy
Total N	25·03 ± 0·28 (8)	21·04 ± 0·39 (9)	29·41 ± 0·13 (2)
Soluble in 1·25 M-KCl	14·40 ± 0·30 (5)	13·00 ± 0·40 (8)	14·30 ± 0·18 (4)
Pptd. on dilution of the extract	5·68 ± 0·30 (5)	4·24 ± 0·19 (8)	5·95 ± 0·12 (4)
Residue soluble in 0·1 N-NaOH	2·17 ± 0·02 (5)	2·34 ± 0·07 (8)	3·21 ± 0·20 (4)
Insoluble residue	8·03 ± 0·51 (5)	5·55 ± 0·17 (8)	11·85 ± 0·13 (4)

Table 2. *Distribution of protein N as percentage of the total N*

	Oestrus	End of pregnancy	After ovariectomy
1. Soluble in 1·25 M-KCl	57·3 ± 1·9	62·0 ± 1·0	48·4 ± 0·5
2. Pptd. on dilution of the extract	22·5 ± 1·1	20·0 ± 1·0	21·4 ± 1·0
3. Residue soluble in 0·1 N-NaOH	8·4 ± 0·6	11·1 ± 0·4	10·7 ± 0·7
4. Insoluble residue	32·7 ± 1·6	26·2 ± 0·7	40·3 ± 0·5

Values of P are as follows for significance of differences in the fractions between any two groups. NS, Not significant.

Protein fraction	Oestrus – pregnant	Oestrus – ovariectomized	Pregnant – ovariectomized
1	NS	<0·05	<0·01
2	NS	NS	NS
3	<0·05	NS	NS
4	<0·05	<0·05	<0·001

For total N the value of P for significance of differences between groups is <0·001.

Table 3. *Ribonucleic acid and deoxyribonucleic acid of the rat uterus in different states*

Results (mg. of RNA P or DNA P/g. wet wt.) are expressed as means ± S.E.M. The experiments were done on two groups of four uteri in oestrus; on four single uteri at the end of pregnancy; and on three groups of five uteri after ovariectomy.

	Oestrus	End of pregnancy	After ovariectomy
RNA P	0·49 ± 0·033	0·34 ± 0·004	0·38 ± 0·049
DNA P	0·38 ± 0·004	0·15 ± 0·003	0·97 ± 0·01
RNA/DNA	1·29	2·34	0·39

neglected. The chopped tissue (weighed samples of about 100 mg. wet wt.) was heated in sealed tubes with 3 ml. of 6 N-HCl for 48 hr. at 100° without preliminary separation of the collagen.

Animals. The rats were piebald Norwegians weighing 170–250 g. Only virgins were used for the normal, non-pregnant uteri and the uteri after ovariectomy. The former were selected all at the stages of mid to late oestrus, by means of vaginal smears characterized by abundant cornified cells (Long & Evans, 1922). The ovariectomized animals were used 20–22 days after the operation; vaginal smears were taken daily for 5 days before use, to ascertain that no oestrous cycle occurred.

RESULTS

In Table 1 the protein N of the four fractions is given per g. wet wt. of the organ in different states. Since the total protein N is different in the three states, a more revealing picture of the changes is obtained by expressing the results as percentages of the total protein N, and this is done in Table 2. It is then seen that the amount of protein extractable by salt solution is significantly greater in the oestrous and pregnant uteri than in the uteri after ovariectomy. The connective tissue residue is greatest after ovariectomy and least in pregnancy, the differences being significant.

Table 3 shows the content in RNA P and DNA P; the marked changes in amount of DNA present/g. wet wt. would be expected to lead to differences in the DNA content of the dilution precipitate made from uteri in different states. For this reason a number of estimations were made of the total nucleic acid P in these precipitates. The values obtained for oestrus, end of pregnancy and after ovariectomy were 0·475 ± 0·014, 0·20 ± 0·019 and 0·83 ± 0·04 respectively. This nucleic acid was mainly DNA; when a separation was made, only 10–20 % appeared in the RNA fraction. It seemed desirable to make a closer assessment of the part of the dilution precipitate contributed by actomyosin and the part contributed by nucleoprotein. Two methods were tried.

(1) If the tissue is thoroughly extracted with a dilute salt solution (0·1 M-KCl buffered with 0·03 M potassium phosphate or sodium borate, pH 7·1) the water-soluble sarcoplasmic proteins are extracted together with the cell particles. An extract of the residue made with 0·1 N-NaOH would then contain the N of the nuclei and of the myofibrillar protein. If the N contribution from the nuclei is known, and this could be determined separately (see Robinson, 1952*b*), it should be possible to arrive at a figure for the actomyosin content. This method was unsuccessful, since many extractions with repeated grindings did not remove more than 30–40 % of the RNA from the residue. Thus in one case from uteri in oestrus the residue contained 0·38 mg. of DNA P and 0·158 mg. of RNA P after seven extractions and grindings. It is likely that some of this remaining RNA was contained in small cell particles, in which

Table 4. *Assessment of the actomyosin-like protein of the dilution precipitate by correcting for nucleoprotein*

Method of obtaining the correction is described in the text. The experiments were done on four groups of four to six uteri in oestrus, on six single uteri in pregnancy and on three groups of nine to twelve uteri after ovariectomy. Results (as means ± S.E.M.) are expressed in mg./g. wet wt. of original tissue.

				Statistical significance of differences between groups (P values)	
	Oestrus	End of pregnancy	After ovariectomy	Oestrus – ovariectomized	Pregnant – ovariectomized
Nucleic acid P in dilution ppts.	0·48 ± 0·014	0·20 ± 0·019	0·83 ± 0·04	—	—
Acid-insol. N in dilution ppts.	5·91 ± 0·13	4·31 ± 0·22	5·95 ± 0·12	—	—
Acid-insol. N after correction for nucleoprotein N	4·72 ± 0·14	3·82 ± 0·28	3·91 ± 0·26	—	—
Protein N after correction, in percentage of total protein N	18·8 ± 0·55	18·2 ± 1·3	13·3 ± 0·93	<0·01	<0·05

the protein N associated with the P might be as high as 17:1 (Hogeboom, Schneider & Pallade, 1948) on analogy with liver-cell particles. The error on the actomyosin due to this would be too large and uncertain and this method was abandoned.

(2) The method adopted for correcting for the nucleic acid in the dilution precipitates was as follows. Nucleoprotein precipitation, in the form of stringy clots, was observed to begin at much lower dilution than was necessary to obtain the main bulk of the precipitate, which was softer and finely divided. In several experiments therefore the usual routine was followed but dilution was carried to the point (0·4–0·3 M-KCl) at which the clots appeared in amount just enough to make their analysis possible. About 50 % of the total nucleic acid was obtained in such clots, which were removed and analysed for total N (protein plus nucleic acid) and for nucleic acid P. The ratio N/P was found to be about 2·5. In some cases this precipitation was carried out in the presence of 5×10^{-3} M ATP, to minimize the precipitation of actomyosin (cf. Hoffmann-Berling, 1956). If it is assumed that this same ratio applies throughout precipitation, and that the protein of the nucleoprotein precipitated contains no actomyosin, then the amount of actomyosin N in a dilution precipitate can be taken as

(mg. of total N of precipitate) – (mg. of N equal to 2·5 × mg. of nucleic acid P of precipitate).

This correction is a maximum one. If only actomyosin itself combines with the nucleic acid, then the correction would consist of the nucleic acid N only – about 1·6 times the nucleic acid P (Chargaff, 1955). The true correction may lie between these extremes. In Table 4 experiments are shown in each of which the nucleic acid P was estimated in the dilution precipitate, and in each the maximum correction has been applied. When this was done, the actomyosin content after ovariectomy was about 72 % of that in oestrus or pregnancy; with smaller corrections the difference would be less.

Table 5. *Collagen content of the rat uterus in different states*

Results (mean values ± S.E.M.) are calculated from the hydroxyproline content of the whole tissue, as described in the text. Four groups of three uteri were used in oestrus, two single uteri in pregnancy; after ovariectomy, two groups of two and one single uterus were used.

	Oestrus	End of pregnancy	After ovariectomy
Collagen (mg./g. wet wt.)	38·3 ± 1·89	23·8 ± 0·75	65·9 ± 1·33
Collagen (% of the connective tissue)	83	71	92

Telfer (1953) has remarked upon the effect of oestradiol injection in bringing about increase in the protein fraction extractable by water from the uterus of the castrated rat. This increase in water-soluble proteins on passing from the castrate to the oestrous and pregnant uterus is seen in our experiments, when the protein precipitated on dilution is subtracted from the protein soluble in salt solution and the result is expressed in percentage of the total proteins; the values come out as 35, 42 and 28 % respectively. The correction to be applied for any water-soluble protein precipitated as nucleoprotein is small, as can be seen from the calculations given above, and would not appreciably alter the picture.

A few experiments were done, with oestrous and pregnant uteri, in which the tissue was extracted three times with 0·5 M-KCl containing 0·05 M potassium phosphate, pH 7·5, the extractant commonly used for extraction of actomyosin from skeletal muscle. The dilution precipitate was obtained as is also usual in actomyosin preparations from skeletal muscle: by dilution with water only to 0·03 M-KCl at pH 6·5–6·8. The results, after correction for nucleoproteins in the way described, were about 75 % of the values obtained by precipitation with 15 % alcohol at 0·1 M-KCl. The precipitate in water was very finely divided, and did not settle or

centrifuge down easily; it was much more difficult to deal with in a quantitative manner than the more flocculent precipitate in 15% alcohol.

Some estimations were made of the collagen content of the uterus in the three states, and these are shown in Table 5.

DISCUSSION

When the results are expressed as percentages of the total nitrogen (Table 2) the main differences to be seen in the protein fractions, by comparing the different states of the uterus with the oestrous state, are: (1) a decrease in the amount of protein extracted by the salt solution after ovariectomy and an increase at the end of pregnancy; (2) a marked increase in the insoluble connective-tissue residue after ovariectomy and a decrease at the end of pregnancy. A systematic study of the changes in wet and dry wt. of the uterus was not made in this work; but the average weight of the uterus in mid-oestrus was 0·4 g. (dry wt. 20%), at the end of pregnancy about 2 g. (dry wt. 18%) and after ovariectomy 0·08 g. (dry wt. 23%). It is thus clear that there were large absolute changes in all the fractions.

The amount of collagen found at the end of pregnancy (23·8 mg./g. wet wt.) is in agreement with that calculated from the figures of Harkness & Harkness (1954) (23·3 mg./g. wet wt.). Our figure for the uterus in oestrus (38·3 mg./g. wet wt.), on the other hand, is higher than the value from the data of Harkness & Harkness (28·3 mg./g. wet wt.). This discrepancy may be due to the use of uteri at different stages of the oestrous cycle. According to Astwood (1939), both the wet wt. and the dry wt. of the rat uterus fall by about 20% during the period between proestrus–oestrus and late oestrus. If, as seems likely, the collagen content per uterus is unchanged during the oestrous cycle, then the amount of collagen/g. wet wt. would be markedly lower early in the oestrous cycle than later, at the time when our specimens were taken.

The collagen content, calculated on the assumption that all the hydroxyproline is derived from collagen, falls short of the amount of insoluble connective-tissue residue (see Table 5). This is probably to be explained by the presence of some elastin. The hydroxyproline content of rat elastin is 2·3%, to be compared with 13·4% in collagen (Neuman & Logan, 1950).

The fraction of protein insoluble in salt solution but soluble in 0·1N-NaOH is of unknown composition. It probably contains material from nuclei remaining in the residue, since only about 80% of the DNA is extracted by the salt solution. In the uteri from ovariectomized animals a large part of the fraction could be accounted for in this way. This explanation cannot apply to the uteri in oestrus, and still less to the pregnant uteri, because of the smaller nucleic acid content/g. wet wt. Unexpectedly, the fraction soluble in sodium hydroxide in percentage of the total nitrogen is significantly higher in pregnancy than in oestrus, indeed as high as after ovariectomy. Robinson (1952*a*), in his study of the proteins of embryonic chick muscle, found this fraction to be as much as 25% of the total nitrogen at hatching; he has suggested that it might include mucopolysaccharides.

It is clear from Table 4 that with the methods used here the changes in 'actomyosin' content of the rat uterus are not large even when expressed as percentage of the total protein. Csapo found a greater change in the rat by the viscosimetric method (a rise of 100%/g. wet wt. by the end of pregnancy). In the rabbit this increase was not so marked, but after ovariectomy a fall from 13 to 6 mg. of actomyosin/g. wet wt. was found. Further investigations are needed to explain the differences in these results.

The degree of homogeneity of the dilution precipitates from the uterus, and the degree of correspondence between the actomyosin in them and skeletal-muscle actomyosin are matters still uncertain (see Needham & Cawkwell, 1956). These questions are under study with uteri from larger animals.

The absolute values given by Telfer for RNA and DNA in the ovariectomized rat are 4·1 and 14·8 mg./g. wet wt. respectively. Conversion of our figures for nucleic acid phosphorus into nucleic acid, on the basis of a phosphorus content of 9·4% in RNA and 9·9% in DNA (Leslie, 1955), gives 4·05 and 9·81 mg./g. wet wt. respectively. The discrepancy in the value for DNA probably depends on the different methods of estimation used. It has been pointed out (Drasher, 1953*b*) that in some tissues too low results may be obtained by the Schmidt & Tannhauser method, owing to appearance of some degraded DNA in the RNA fraction. However, if this were so in the present work, one would expect the RNA value to be high. On the other hand, too high values for DNA can be obtained by the Dische method (used by Telfer) in the presence of protein (Dounce, 1952). A similar discrepancy between the results on the mouse, obtained by Drasher (using the Schmidt & Tannhauser method) and by Jeener (using the Dische method), leads us to suspect that the former is the more reliable with these tissues.

The experiments of Jeener (1948) and Drasher (1952) on the mouse and of Telfer (1953) on the rat show that injection of oestradiol into the castrated animal leads to a marked increase in the RNA/DNA ratio of the uterus. In Jeener's work injection of 20 μg. led in 24 hr. to an increase from 0·28 to 0·62; the wet wt. rose by 200% and the dry wt. by 100%

but there was little mitosis. Thus growth of the uterus after such treatment, as well as during the oestrous cycle (Drasher, 1953*a*), seems to depend largely on increase in cell size reflected in increase in cytoplasmic RNA relatively to nuclear DNA.

Increase in the cell dimensions is also recognized as the main basis of growth of the uterus during pregnancy (Reynolds, 1949), and in this connexion the work of Drasher (1953*b*) may be mentioned. In her experiments following the changes in RNA, DNA and protein nitrogen throughout pregnancy in the mouse, the ratios in oestrus, after ovariectomy and at the end of pregnancy were 1·58, 0·43 and 1·12 respectively. The first two values are similar to those of the present work (1·29 and 0·39), but the last is much lower than the 2·34 found by us at the end of pregnancy. It is interesting to notice that in available curves for increase in weight during pregnancy in the rat uterus (Siegmund, 1930; Harkness & Harkness, 1954) growth is shown as continuing at a steady rate from the 13th to the 20th day. In Drasher's curves for the mouse there is a sudden increase in protein nitrogen at the 11th day, and from the 13th to the 20th day further increase is much slower. It is unlikely that there is any fundamental difference in the growth pattern of the pregnant uterus between the rat and the mouse, but it is possible that the rate of growth during the last week can be affected by conditions such as the nutritional state, number of foetuses, etc. A study for the rat of the RNA/DNA ratio in the uterus, correlated with growth rate, would be of great interest.

SUMMARY

1. Four protein fractions, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) have been estimated in the rat uterus in mid-oestrus, at the end of pregnancy and after ovariectomy. The fractions were: (i) the protein soluble in 1·25M potassium chloride solution; (ii) the protein precipitated on dilution of this extract; (iii) the fraction of the residue soluble in 0·1N sodium hydroxide solution; (iv) the insoluble residue.

2. When the results are expressed as percentages of the total protein, the most important changes in the protein fractions were a significant fall in (i) and rise in (iv) after ovariectomy, as compared with the oestrous state; and a significant rise in (i) and fall in (iv) by the end of pregnancy, as compared with the oestrous state. When certain corrections were made there was significantly less of fraction (ii) after ovariectomy than in the other two states.

3. The RNA/DNA ratios in mid-oestrus, at the end of pregnancy and after ovariectomy were 1·29, 2·34 and 0·39 respectively.

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The Distribution and Properties of Transhydrogenase from Animal Tissues

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In 1952 Colowick, Kaplan, Neufeld & Ciotti described a new enzyme which they named pyridine nucleotide transhydrogenase. This enzyme, which was extracted from *Pseudomonas fluorescens*, catalyses the following reaction:

$$TPNH + DPN^+ \rightleftharpoons TPN^+ + DPNH,$$

where TPN^+ and TPNH represent the oxidized and reduced forms of triphosphopyridine nucleotide and DPN^+ and DPNH represent the corresponding forms of diphosphopyridine nucleotide. Further studies by Kaplan, Colowick & Neufeld (1952, 1953) showed that the reactions were brought about by hydrogen, not electron, transfer and that the enzyme occurred in some animal tissues.

The purpose of the present study was to examine more closely the distribution in animal tissues and also to obtain some information on the nature of the enzyme.

EXPERIMENTAL

Materials

Animals. Earthworms, crabs and opossums were collected from their natural environment and killed at the laboratory. Rats and rabbits were obtained from laboratory animal houses. Slaughterhouse material was used for studies of chickens, pigs, oxen, sheep and horses; tissues from these animals were brought to the laboratory in ice-filled Dewar flasks, the transport time being always less than 2 hr.

Chemicals. DPN^+ and DPNH were purchased from Boehringer and Sons, Mannheim; purities were determined by alcohol dehydrogenase, DPN^+ assaying 95–100% and DPNH 54–67%. TPN^+ (72%) was kindly given by Professor F. Dickens, and some samples (78–80% pure) were purchased from Sigma Chemicals, Inc., U.S.A. (assayed by *iso*citric dehydrogenase). DL-*iso*Citric lactone was purchased from L. Light and Co., and converted into *iso*citrate by the method of Krebs & Eggleston (1944). Flavine mononucleotide (FMN) was presented by Sigma Chemicals and flavin-adenine dinucleotide by Dr L. G. Whitby, Cambridge. Digitonin was purchased from L. Light and Co., and British Drug Houses Ltd.

*Enzymes. iso*Citric dehydrogenase was prepared by the method of Grafflin & Ochoa (1950). Crystalline alcohol dehydrogenase was purchased from Boehringer and Sons or isolated according to Racker (1950).

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Methods

All manipulations were conducted at 0–2°.

Particles. The 'KCl particles' of Kaplan *et al.* (1953) were prepared by blending tissue with 10 vol. of 0·9% KCl in 0·01 M-$NaHCO_3$ for 2 min., centrifuging for 5 min. at 400 *g* and decanting the supernatant through two layers of coarse muslin. This fluid was then centrifuged for 20 min. at 20000 *g*. The sedimented particles were suspended in 1 ml. of 0·9% KCl for each g. of tissue originally blended.

Mitochondria. These were isolated by grinding tissue in 5 vol. of 0·25 M sucrose in a Potter–Elvehjem homogenizer. The suspension was poured through muslin and centrifuged for 10 min. at 700 *g*. The residue (nuclear fraction) was washed with 5 vol. of sucrose and the combined supernatants were centrifuged for 20 min. at 5000 *g*. The mitochondrial fraction was washed once with 5 vol. of sucrose and suspended in 1 ml. of 0·9% KCl for each g. of tissue.

Digitonin–calcium phosphate extract. This was prepared according to Kaplan *et al.* (1953) with minor modifications. The digitonin–particle suspension was centrifuged for 20 min. at 25 000 *g* and the digitonin–calcium phosphate mixture for 15 min. at the same speed. The gel from 20 g. of tissue was washed with 4 ml. portions of M-K_2HPO_4 and eluted with 3 ml. portions of M-KH_2PO_4.

Ammonium sulphate fractionation. The solid salt was added to give the required concentration and M-$NaHCO_3$ was used to maintain the pH.

Assays. These were all conducted at room temperature (18–23°). The experiments on distribution were carried out according to Kaplan *et al.* (1953) by following the reaction $TPNH + DPN^+ \rightarrow TPN^+ + DPNH$ by means of the increase in $E_{340\,m\mu}$. Each 1 cm. cell contained 0·25 ml. of TPN^+ (1 mg./ml.), 0·1 ml. of KCN (2 mg./ml.), 0·1 ml. of nicotinamide (73 mg./ml.), 1·5 ml. of 0·16 M aminotrishydroxymethylmethane (tris) buffer, pH 7·5, and 0·2 ml. of *iso*citric dehydrogenase (sufficient to give instantaneous reduction of the TPN^+); the blank received 0·6 ml. of water and the experimental cell 0·6 ml. of *iso*citrate (1·5 mg./ml.). To both cells was then added 0·2 ml. of the preparation to be tested and when the $E_{340\,m\mu}$ became constant (1–5 min.) 0·2 ml. of DPN^+ (2 mg./ml.) was added. With active preparations the $E_{340\,m\mu}$ increased because of the accumulation of DPNH by transhydrogenase action and the instantaneous regeneration of TPNH by the *iso*citric system. Nicotinamide inhibits nucleotidases and KCN prevents reoxidation of coenzymes by the cytochrome system.

When using KCl particles one of the units defined by Kaplan *et al.* (1953) was chosen, i.e. 100 times the change in $E_{340\,m\mu}$ (between 1 and 7 min. after DPN^+ addition)/g. of tissue. It was not feasible to use this unit in studies with mitochondria and purified extracts and in these cases the

unit was taken as μg. of DPNH formed/mg. of enzyme protein/hr. It is these units which are referred to as activity. The amount of enzyme was given by the amount of coenzyme which could be oxidized in 1 hr. by the whole fraction. Protein in enzyme preparations was estimated by observing the $E_{280\ m\mu}$ after solution in 0·1 N-NaOH. It was arbitrarily assumed that the enzyme had the same absorption characteristics as bovine serum albumin, i.e. that a solution containing 1·5 mg./ml. gave an $E_{280\ m\mu}$ of 1·0 in a 1 cm. cell.

The method given above was not suitable for studying inhibitors because these compounds often acted on the *iso*-citric dehydrogenase, thus obscuring their effect on transhydrogenase. Therefore the reaction

$$DPNH + TPN^+ \rightarrow DPN^+ + TPNH$$

was studied. To conserve some reagents, 0·2 cm. cells were used and each contained 0·1 ml. of enzyme in M phosphate buffer, pH 6·3, and 0·05 ml. of TPN^+ (1 mg./ml.); the blank received 0·3 ml. of water, the control 0·2 ml. of water plus 0·1 ml. of DPNH (0·3 mg./ml.), and the experimental cell 0·2 ml. of inhibitor (adjusted to pH 6·3) plus 0·1 ml. of DPNH. The reaction was allowed to proceed for 6 min. and then 0·025 ml. of tris [saturated solution in 25% (v/v) ethanol] was added. This not only stopped the reaction but provided a suitable pH (10) and substrate (ethanol) for the action of alcohol dehydrogenase (0·06 ml. of 0·5 mg./ml.) which was next added. The increase in $E_{340\ m\mu}$ was noted until it became constant (1–3 min.); this gave the amount of DPN^+ previously formed. Finally 0·01 ml. of DPN^+ (2 mg./ml.) was added to check the efficiency of the DPN^+ determination, particularly with regard to the possible action of an inhibitor on alcohol dehydrogenase. Where less than 90% recovery of the DPN^+ was obtained, the experiment was rejected. The only inhibitors used which affected alcohol dehydrogenase were those which reacted with sulphydryl groups. In these cases, an excess of glutathione was added immediately after stopping the transhydrogenase reaction. Because the DPNH samples sometimes contained up to 10% of DPN^+, it was necessary to apply a small correction for the amount actually found by analysis.

RESULTS

Distribution

Table 1 shows the distribution of transhydrogenase in different tissues of several animals as exemplified by the activity of KCl particles. Muscle, and in particular, heart muscle, was the only type of tissue found to give high activity. In some cases (rat kidney and liver) there was a decrease in $E_{340\ m\mu}$ after the addition of DPN^+, leading to negative values for the activity. This was probably due to the presence of systems capable of oxidizing the reduced coenzymes without the mediation of the cytochrome system. Such results are to be expected when crude preparations are used. Therefore, the distribution studies were extended to determine if activity were localized in some subcellular unit. Table 2 shows that it is the mitochondria which contain the transhydrogenase, and Table 3 compares the activity of mitochondria from different tissues. Again heart muscle was most active and tissues which previously gave negative values (rat kidney and liver) now showed moderate activities. All the results given below were obtained with rat heart; similar results were obtained with ox heart.

Table 1. *Activity of* KCl *particles*

The values are means. Units of activity are 100 × the change in $E_{340\ m\mu}$/6 min./g. of tissue.

Tissue	Activity	No. of observations
Rat brain	4	5
Rat heart	87	14
Rat kidney	Negative	2
Rat liver	Negative	3
Rat muscle	28	2
Rat spleen	4	3
Rat testis	9	2
Pig heart	36	3
Ox heart	50	4
Sheep heart	22	1
Horse heart	20	2
Opossum heart	67	1
Chicken heart	125	1
Chicken liver	4	1
Crab muscle	5	2
Earthworm muscle	7	1

Zero values were given by rat prostate and seminal vesicle, pig brain, calf brain, ox brain, sheep brain, and rabbit brain and liver.

Table 2. *Subcellular distribution in rat tissues*

Values are μg. of DPN^+/mg. of protein/hr.

	Nuclei	Mitochondria	Soluble fraction
Brain	0	2	0
Heart	5	47	4
Kidney	6	27	0
Liver	2	13	0
Spleen	2	8	3

Table 3. *Activity of mitochondria*

Values are means as μg. of DPN^+/mg. of protein/hr.

Tissue	Activity	No. of experiments
Rat brain	4	2
Rat heart	68	3
Rat kidney	16	3
Rat liver	13	1
Rat muscle	11	1
Rat spleen	4	2
Rat testis	4	1
Rabbit heart	33	2
Rabbit kidney	5	1
Pig heart	15	3
Horse heart	21	1

Purification

Transhydrogenase could be extracted from fresh KCl particles or mitochondria by grinding with glass powder and 0·9% KCl containing 0·005 M

phosphate buffer, pH 6·9. There was only a slight increase in activity. If frozen-dried KCl particles or mitochondria were stirred with KCl–phosphate the resulting extract showed about double the activity of the original material and these extracts could be fractionated with ammonium sulphate (Table 4), giving, between 20 and 35 % saturation, good yield and increased activity. The 20–35 % fraction could be reprecipitated with little loss of material and a further gain in activity, e.g. a 20–35 % fraction of 318 units of activity (μg. of DPN^+/mg. of protein/hr.) on reprecipitation between 20 and 35 % saturation had an activity of 524 units with 81 % recovery of the original amount of enzyme.

All these preparations were unstable, losing 25–30 % of their activity overnight at 0° or −10°. Re-activation could not be obtained by adding glutathione, flavine mononucleotide or flavine adenine dinucleotide; nor could activity be preserved by storage in 1 % albumin, one-tenth saturated ammonium sulphate or 0·0013 M glutathione. The effect of pH on the activity of transhydrogenase was tested by replacing the tris buffer with phosphate (final concentration 0·06 M). Fig. 1 shows the curve obtained, the pH values plotted being those determined at the end of the experiment; the pH optimum was 6·3 and this was used in subsequent experiments. The same optimum was found for the reaction $DPNH + TPN^+ \rightarrow DPN^+ + TPNH$ (Fig. 1).

Kaplan *et al.* (1953) extracted transhydrogenase from KCl particles by adding digitonin and purified the extract by adsorption on calcium phosphate gel and ten elutions with M-KH_2PO_4. Table 5 shows that most of the enzyme could be obtained with only six elutions and that on storage overnight there were losses of up to 30 % in activity. In this experiment 30 % of the amount of enzyme in the digitonin extract was not adsorbed by the gel, and of the amount adsorbed 19 % was not recovered in the seven eluates. In a further experiment, six active eluates were combined and had a pH of 6·6. Overnight at 0° there was 45 % loss of activity; at −10° with no addition there was 56 % loss, in 1 % albumin 45 %, in one-tenth saturated ammonium sulphate 80 %, and in 0·0013 M glutathione 50 %. The effect of pH on storage was investigated by combining six active eluates and adjusting samples to different pH values. After storage overnight the samples were re-adjusted to pH 6·3, centrifuged and activities determined (Table 6). It can be seen that 6·3 was the optimum pH for storage, the increase in activity probably being due to removal of inert proteins. Further, such solutions could be fractionated with ammonium sulphate between 20 and 35 % saturation to give increases in activity of up to 50 %. Also the activity could be increased by prolonged centrifuging at high speeds, e.g. 3 hr. at 160 000 *g*;

Table 4. *Fractionation with ammonium sulphate*

Units are μg. of DPN^+/mg. of protein/hr. The mitochondria had an activity of 70 units. They gave a KCl–phosphate extract equal to 125 units with an amount of enzyme equivalent to 4·88 mg. of DPN^+/hr.

Fraction (saturation with $(NH_4)_2SO_4$, %)	Activity (μg. of DPN^+/mg. of protein/hr.)	Amount of enzyme (mg. of DPN^+/hr.)
0–10	483	0·41
10–20	248	0·18
20–30	381	1·78
30–35	248	1·13
35–40	16	0·03
		Sum 3·53
		(72 % recovery)

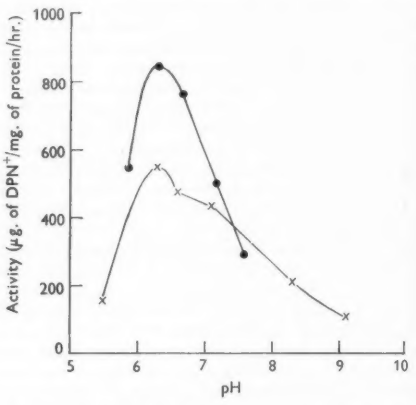

Fig. 1. Effect of pH on transhydrogenase activity of rat heart. ●, $TPNH + DPN^+ \rightarrow TPN^+ + DPNH$; ×, $DPNH + TPN^+ \rightarrow DPN^+ + TPNH$. Experimental conditions were as described in the text under Methods. It should be noted that the conditions are not directly comparable.

Table 5. *Activity of eluates from calcium phosphate gel*

The eluates were stored at −10° for 16 hr.

Eluate no.	Activity (μg. of DPN^+/mg. of protein/hr.)	Amount of enzyme (mg. of DPN^+/hr.)	Activity after storage (μg. of DPN^+/mg. of protein/hr.)
1	148	6·9	124
2	360	14·4	298
3	551	14·4	427
4	1030	12·2	710
5	1070	8·9	759
6	1120	4·1	888
7	250	1·5	186

the eluates were usually light golden in colour but after such centrifuging the contents of the tube could be drawn off in several fractions (Table 7) ranging from colourless to brown. The increased activity of the top and intermediate fractions was probably due to removal of the coloured impurities; the extent of this increase in different experiments varied between 100 and 500%.

Inhibitors

Studies with inhibitors were made both with combined eluates and also with the fractions prepared by high-speed centrifuging of these eluates. The results in Table 8 show that sulphydryl reagents were powerful inhibitors.

Table 6. *Effect of* pH *and storage on activity*

Values give the activity compared to the original combined eluates taken as 100.

pH	Storage temp. 0°	Storage temp. −10°
5·8	75	66
6·3	132	108
7·1	60	45
8·0	0	0
8·3	12	24

Table 7. *Separation of transhydrogenase by centrifuging*

Combined eluates (30 ml.; activity, 209 units) were centrifuged for 3 hr. at 160 000 *g*.

Fraction	Volume (ml.)	Colour	Activity (μg. of DPN^+/mg. of protein/hr.)
1	2·4	Colourless	358
2	2·4	Colourless	560
3	5·0	Colourless	489
4	5·1	Faint yellow	692
5	5·2	Faint yellow	319
6	7·5	Yellow	124
7	1·9	Yellow-brown	89
Residue	—	Brown	—

Table 8. *Effect of inhibitors on transhydrogenase*

Figures give the percentage of the control activity.

Inhibitor	Concn. (mM)	Activity (% of control)
Fluoride	10	99
Pyrophosphate	2	91
Pyrophosphate	10	53
Azide	9	105
o-Phenanthroline	5	96
Diethyldithiocarbamate	9	105
Glycine	9	94
Oxalate	9	96
Cyanide	1	100
p-Chloromercuribenzoate	2·2	68
p-Chloromercuribenzoate	4·5	14
Iodosobenzoate	0·17	79
Phenyl mercuriacetate	0·03	33
$HgCl_2$	0·05	65
Arsenite	1·0	75

Michaelis constants

These were determined for the reaction

$$DPNH + TPN^+ \rightarrow DPN^+ + TPNH$$

by the assay method used for studying inhibitors. The values found were $1{\cdot}4 \times 10^{-5}$ M DPNH and $15{\cdot}7 \times 10^{-5}$ M TPN^+.

Absorption spectra

All preparations showed a maximum at 275 mμ. and unpurified preparations also had a small peak at 407 mμ. which was probably due to contaminating methaemoglobin.

DISCUSSION

The distribution reported is similar to that observed by Kaplan *et al.* (1953), i.e. heart being most active and some tissues entirely inactive, but these workers found higher activities than those reported here. The localization of transhydrogenase in mitochondria is in keeping with the importance of these particles in metabolism.

Most of the enzyme preparations had a faint yellow colour and some green fluorescence indicative of flavines. However, since no flavine bands were present in the absorption spectra and since colourless, active solutions could be obtained by means of high-speed centrifuging, the enzyme is probably not a flavoprotein. Further, aged preparations could not be re-activated by adding flavine mono- or dinucleotide. Also attempts to split off a prosthetic group by means of ammonium sulphate at pH 3 and 0° were unsuccessful; the supernatants obtained were clear, colourless and non-fluorescent. The marked inhibition brought about by various sulphydryl reagents shows that some intact sulphydryl groups are necessary for activity. The lack of inhibition by metal-combining reagents suggests that the enzyme is not a metalloprotein.

The pH optimum of 6·3 agrees well with the 6·2 found by Kaplan *et al.* (1952). An attractive hypothesis for the function of transhydrogenase would be that its presence allows cells to make greater use of systems requiring triphosphopyridine nucleotide. Most tissues have much less $TPN^+ + TPNH$ than $DPN^+ + DPNH$ (Glock & McLean, 1955), and in many cases the amount of $TPN^+ + TPNH$ is so small that it is questionable whether systems requiring triphosphopyridine nucleotide can play a quantitatively significant role. If it could be shown that tissues rich in transhydrogenase had a high $TPN^+/TPNH$ ratio, and that tissues poor in transhydrogenase had a low value,

then the role of transhydrogenase would be clearly indicated. However, the evidence for this is not definite. Rat heart muscle, kidney and liver, which all have high transhydrogenase activities, contain 3–6 μg. of TPN^+/g. but the concentration of TPNH is 33–205 μg./g., the TPN^+/TPNH ratio being 0·03–0·13 (Glock & McLean, 1955). Rat prostate, seminal vesicle, spleen and testis, which have negligible transhydrogenase activity, contain negligible quantities of TPN^+ ($< 2\,\mu$g./g.) and only 8–12 μg. of TPNH/g. It could be argued that transhydrogenase occurs along with the higher concentrations of TPN^+ + TPNH; on the other hand, it would be expected that the presence of transhydrogenase would lead to a value for TPN^+/TPNH higher than that observed (0·03–0·13), especially since all tissues so far tested contain more DPN^+ than DPNH, e.g. Glock & McLean (1955) give DPN^+/DPNH for rat heart as 1·6 and Jedeikin & Weinhouse (1955) give 5·5 for the same tissue, the total concentrations given being respectively 483 and 563 μg./g.

SUMMARY

1. Transhydrogenase occurs in animal tissues, particularly in heart muscle and to a smaller extent in skeletal muscle, kidney and liver. Other tissues such as brain, prostate, seminal vesicle, spleen and testis show small or negligible activities.

2. Transhydrogenase is localized in mitochondria.

3. Transhydrogenase can be purified by precipitation with ammonium sulphate in the range 20–35% saturation.

4. Inert material can be removed from solutions of transhydrogenase and the activity thus increased, by centrifuging for 3 hr. at 160 000 *g*.

5. The pH optimum for each of the reactions

$$TPNH + DPN^+ \rightarrow TPN^+ + DPNH$$

and

$$DPNH + TPN^+ \rightarrow DPN^+ + TPNH$$

is 6·3.

6. Michaelis constants for the reaction

$$DPNH + TPN^+ \rightarrow TPNH + DPN^+$$

are $1{\cdot}4 \times 10^{-5}$ M DPNH and $15{\cdot}7 \times 10^{-5}$ M TPN^+.

7. The action of transhydrogenase is strongly inhibited by sulphydryl reagents.

8. Solutions of transhydrogenase show maximum light absorption at 275 mμ.

9. There is no evidence at present for the presence of any flavine or metal prosthetic group in the enzyme.

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The Utilization of Inorganic Sulphate by Granulation Tissue

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In a preceding paper (Kodicek & Loewi, 1955), the uptake of $^{35}SO_4^{2-}$ by granulation tissue *in vivo* and *in vitro* was described. It was found that a sulphated mucopolysaccharide akin to chondroitin sulphate was formed, the formation depending on enzymic activity of the tissue.

The present communication is concerned with studies of the relationship between the respiratory activity of granulation tissue at various stages of maturation and the uptake of $^{35}SO_4^{2-}$. The effect of various added substances has also been investigated. We have examined the inhibitory effects on the system of various phenols, some sulphated substances and fluoroacetate. Addition of glucose to respiring sections of cartilage (Boyd & Neuman, 1954; Boström & Jorpes, 1954) resulted in increased incorporation of $^{35}SO_4^{2-}$, and Boström, Roden & Vestermark (1955) reported that glutamine acted as an accelerator of chondroitin sulphate synthesis in cartilage.

Since granulation tissue possesses a system for the synthesis of sulphated mucopolysaccharides, we thought it of interest to determine whether this tissue would also sulphate phenols. Such activity

has been shown in liver slices by De Meio & Arnolt (1944), but Segal (1955) was unable to show this activity in preparations of rat spleen, heart, kidney or hepatoma.

METHODS

Material. Granulation tissue was obtained from guinea pigs by tendonectomy as previous described (Kodicek & Loewi, 1955). The tissue was sliced thinly with a razor blade and distributed in flasks so that the tissue from the same animal furnished both test and control. This is essential in view of the great variation among individual guinea pigs. Radioactive sulphate used was carrier-free $Na_2{}^{35}SO_4$, supplied by Radiochemical Centre, Amersham, Bucks.

Incubation procedures. (i) Tissue slices were incubated in Krebs–Ringer–phosphate (Krebs, 1950), containing 10^{-2} M glucose (except in the few cases denoted where a glucose-free medium was used). To each flask, containing 4 ml. of medium, 4 μc of $Na_2{}^{35}SO_4$ was added. Substances under test were added dissolved in 0·1 ml. of medium and neutralized (pH 7·4) where necessary. Incubation was carried out in Warburg flasks at 37° with O_2 as gas phase. At the end of incubation (4 hr.) the tissue slices were washed as previously described (Kodicek & Loewi, 1955), dried and weighed and the radioactivity was assayed. In general, experiments were carried out in duplicate.

(ii) In experiments on phenyl sulphate synthesis, incubation was performed as above, in the presence of phenol or *m*-aminophenol at 10^{-3} M (final concentration). Two methods were used to detect the formation of sulphated phenols. In the first, the tissue, after incubation for 4 hr., was removed and the medium was treated with 1 ml. of 20% trichloroacetic acid. To the filtrate, 0·6 ml. of 0·05 N-Na_2SO_4 (non-radioactive) and 0·4 ml. of N-$BaCl_2$ were added and the solution was left at 80° for 0·5 hr. The precipitated $BaSO_4$ was removed by centrifuging and the addition of Na_2SO_4 and $BaCl_2$ was twice repeated. The final supernatant was hydrolysed by the addition of 2 ml. of N-HCl and heating in a boiling-water bath for 15 min. A volume (5 ml.) of 0·05 N-Na_2SO_4 and 3 ml. of N-$BaCl_2$ were added, and the resulting precipitate, after standing at about 80° for 2 hr., was assayed for radioactivity.

Chromatographic analysis. In the second method, involving the above incubation procedure, the sulphated products were detected chromatographically. The medium, after incubation, was extracted three times with ether to remove free phenol. The combined aqueous layers were concentrated to dryness under reduced pressure and the residue was taken up in 96% ethanol. This extract was examined by ascending chromatography for 15–16 hr. on Whatman no. 1 paper with *n*-butanol–ethanol–water (4:1:5, by vol.). After drying at room temperature, the papers were suspended in HCl vapour for 1 min. and then heated at 100° for 2 min. to hydrolyse *O*-sulphates. The phenol thus liberated was identified by spraying the paper with a solution of diazotized sulphanilic acid (Evans, Parr & Evans, 1949). When the chromatogram was finally exposed to NH_3, coupled phenol was visible as an orange-yellow spot (R_F 0·47). Free phenol moved to the solvent front. Unhydrolysed phenyl sulphate gave no colour. Phenyl sulphate, for use as a marker, was prepared by the method of Feigenbaum & Neuberg (1941).

Measurement of radioactivity. (i) Radioactivities of $BaSO_4$ precipitates were counted at infinite thickness with a mica end-window counter tube of diameter 1 in. (General Electric Co., London; type E.H.M.2S). Counts were corrected for decay and expressed in terms of dry wt. of tissue.

(ii) Radioactive components on paper chromatograms were counted by direct application of a Geiger–Müller tube, type E.H.M.2S, with a thin end-window.

RESULTS

Relationship between Q_{O_2}, sulphate uptake and age of tissue. Fig. 1 (*a*) shows nineteen values for Q_{O_2} (μl. of O_2/mg. dry wt. of tissue/hr.) of granulation-tissue slices obtained at varying intervals of time

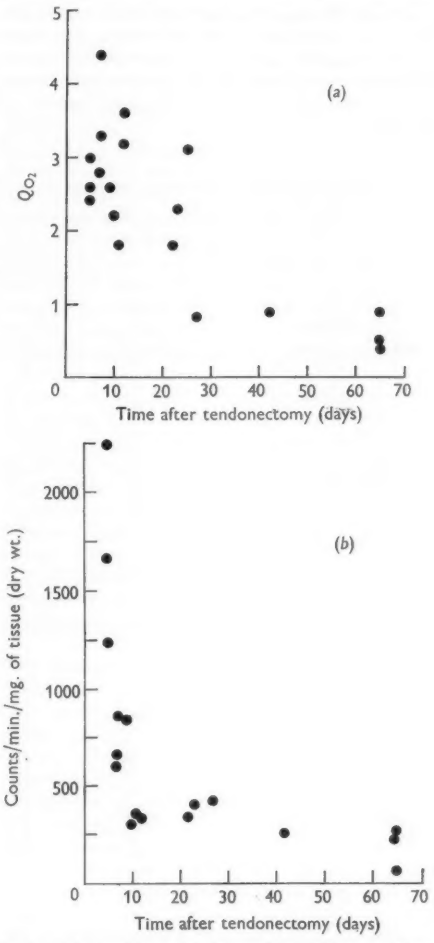

Fig. 1. Relationship of (*a*) Q_{O_2} and (*b*) $^{35}SO_4{}^{2-}$ uptake of granulation-tissue slices to maturation of the tissue.

after tendonectomy in nineteen animals. Though there is a wide scattering of points, in general a decrease with maturation of the tissue can be observed. Fig. 1 (*b*) shows the corresponding values (with two pairs of coincident points) for $^{35}SO_4^{2-}$ uptake, and though it exhibits also a wide scattering of results, it similarly suggests a decrease with maturation of the tissue.

Effect on Q_{O_2} and sulphate uptake of various added substrates. It is clear from the data in Table 1 that although addition of glucose at varying concentrations did not appreciably affect the Q_{O_2}, the uptake of $^{35}SO_4^{2-}$ was increased considerably. Pyruvate similarly caused a rise in the uptake of sulphate, but in this case the Q_{O_2} was increased at the same time. Addition of L-glutamine did not have any effect on Q_{O_2} or sulphate uptake of granulation tissue. Increasing concentrations of fluoroacetate caused a progressive fall in both Q_{O_2} and uptake of sulphate.

The inhibition of sulphate uptake produced by salicylate (Kodicek & Loewi, 1955) and our observation that phenol was sulphated by granulation tissue, led to an investigation of the effects of various phenolic substances. The results are given in Table 2. Phenol, at concentrations of 10^{-3}M and 10^{-2}M, caused no appreciable fall of Q_{O_2} and only a slight decrease of sulphate uptake. At 10^{-1}M, the Q_{O_2} was abolished and the uptake of sulphate almost completely inhibited. It appears therefore that phenol, at concentrations which do not interfere with oxygen uptake, has only a small effect on sulphate uptake. Aminophenol at 10^{-3}M caused a similar slight effect. Inhibition by salicylate, as already reported, was 63% at 10^{-4}M. On the other hand, sodium gentisate at 10^{-3}M did not inhibit sulphate uptake and the Q_{O_2} did not appear to be affected, even at 10^{-1}M.

The effect of phenyl sulphate was considerably greater than that produced by the same concentra-

Table 1. *Effect of various substances on the Q_{O_2} and [^{35}S]sulphate uptake of granulation tissue*

For conditions see incubation procedure (i) under Methods.

		Experiment		Control		
Substance	Concn. (M)	Q_{O_2}	Counts/min./ 100 mg. dry wt.	Q_{O_2}	Counts/min./ 100 mg. dry wt.	Change in $^{35}SO_4^{2-}$ uptake (%)
D-Glucose	2×10^{-2}	−1·8	27 200	−1·8	14 400	+89
	10^{-1}	−1·8	40 800	−1·9	17 000	+140
	2×10^{-1}	−1·7	32 850	−1·6	14 500	+127
Sodium pyruvate	2×10^{-2}	−2·9	42 600	−1·5	17 600	+142
L-Glutamine	10^{-3}	−2·1	92 500	−1·7	88 000	+5
	10^{-2}	−2·0	95 250	−2·3	89 500	+6
Sodium fluoroacetate	10^{-3}	−1·2	43 000	−2·9	88 400	−51
	10^{-2}	−0·7	25 200	−2·5	80 000	−68
	2×10^{-2}	0	2 918	−2·3	79 000	−98

Table 2. *Effect of phenolic substances and some sulphated substances on [^{35}S]sulphate and Q_{O_2} of granulation tissue*

For conditions see incubation procedure (i) under Methods.

		Experiment		Control		
Substance	Concn. (M)	Q_{O_2}	Counts/min./ 100 mg. dry wt.	Q_{O_2}	Counts/min./ 100 mg. dry wt.	Change in $^{35}SO_4^{2-}$ uptake (%)
Phenol	10^{-3}	−3·0	43 375	−3·2	58 050	−15
	10^{-2}	−2·2	30 700	−2·4	38 200	−20
	10^{-1}	0	3 030	−3·0	75 000	−96
Sodium gentisate	10^{-3}	−2·7	27 200	−2·2	20 900	+30
	10^{-2}	−3·1	43 600	−4·4	79 600	−45
	10^{-1}	−3·2	4 560	−2·8	85 200	−95
Phenyl sulphate	10^{-4}	−2·8	23 800	−2·2	28 900	−20
	10^{-3}	−2·1	28 900	−2·6	54 500	−47
	10^{-2}	−3·5	10 500	−2·7	28 800	−64
Potassium methanesulphonate	2×10^{-2}	−1·9	24 400	−2·2	55 650	−56
	4×10^{-2}	−1·0	13 250	−1·9	59 500	−76
Potassium ethyl sulphate	2×10^{-2}	−3·4	30 500	−3·3	28 400	+7

tion of phenol. This suggested that the effect was not due to phenol liberated by hydrolysis during the incubation.

The inhibition of $^{35}SO_4^{2-}$ uptake shown in the presence of phenyl sulphate suggested the trial of other sulphur-containing compounds. Though potassium methanesulphonate produced inhibition, potassium ethyl sulphate, even at 2×10^{-2}M, showed no such effect.

Synthesis of phenyl sulphate by granulation tissue. The data shown in Table 3 suggested that granulation-tissue slices, like liver slices, could sulphate aminophenol, since radioactive sulphate was held in the medium after precipitation of remaining ionic sulphate. Similar results were obtained with phenol, the formation of phenyl sulphate by granulation tissue being shown chromatographically. A coincident coloured and radioactive spot (R_F 0·47) corresponding to phenyl sulphate was obtained. When non-radioactive phenyl sulphate was added to the extract of the medium, the same coincident radioactive and coloured component (R_F 0·47) was observed. In the solvent system used, inorganic sulphate remained almost stationary at the origin. In the presence of liver slices, the results were similar. Approximately 0·2 μmole of phenyl sulphate was formed/100 mg. of granulation tissue in 4 hr. Control experiments performed with boiled-tissue slices and with tissue without added phenol produced no evidence of the formation of phenyl sulphate. In the same way it was found that phenyl sulphate was formed in comparable amounts when slices of calf costal cartilage were incubated with phenol.

Experiments to determine whether salicylate was sulphated by granulation tissue have so far shown no evidence of the formation of a sulphated derivative.

DISCUSSION

Although the Q_{O_2} of granulation tissue is relatively low at all times and the scatter of values is great, a general decline in values with age of the tissue is apparent. The cellularity of granulation tissue shows a marked decrease with age, as the tissue becomes more collagenous. The decline in Q_{O_2}, together with sulphate uptake, serves to emphasize further the dependence of the sulphation processes on cellular activity. The highest value obtained for Q_{O_2} was $-4·4$, a low value when compared with values from tissues such as liver, brain and kidney (Krebs, 1950). Neuhaus (1929) studied granulation tissue from tuberculous lymph nodes. The average Q_{O_2} in ten experiments with such tissue from guinea pigs was $-6·7$. Injection of kieselguhr produced a more cellular granuloma, slices of which had a Q_{O_2} of $-12·9$. Since sections of the granulation tissue studied by ourselves showed large numbers of collagen fibres rather than many cells, the low values for Q_{O_2} are explicable.

The effect of glucose is difficult to interpret. In the presence of this sugar a very considerable increase in sulphate uptake was achieved. Since, however, no values for specific activities are available (the total amounts of sulphate were too small for estimation) it is not possible to decide whether this increase represents a rise in net synthesis of sulphated polysaccharide or only an increase of the degree of labelling. There was no apparent effect on Q_{O_2}, but a small change in these low values might not have been perceptible. It is possible therefore that glucose acted by virtue of its availability as a source of substrate for polysaccharide formation, but it is also possible that it acted by increasing the amount of available energy for the formation of sulphated polysaccharide or sulphate transport into the cell. The present results confirm the findings of Boyd & Neuman (1954) and Boström & Jorpes (1954) in so far as sulphate uptake of cartilage is increased in the presence of glucose.

Addition of pyruvate caused a considerable rise in sulphate uptake and an appreciable increase in Q_{O_2}. It is possible therefore, but not established, that an increased supply of energy caused the raised sulphate uptake. That the uptake of sulphate is an energy-requiring process has already been shown in an earlier paper (Kodicek & Loewi, 1955). Boström *et al.* (1955) have found that L-glutamine acts as an accelerator of chondroitin sulphate synthesis in cartilage. We were unable to detect such an effect with granulation tissue.

The effect of fluoroacetate suggests the importance of the citric acid cycle for the provision of energy necessary for sulphate uptake. It serves, further, to demonstrate that the cycle plays a role in the metabolic pathways of fibroblasts. The effect of salicylate in inhibiting sulphate uptake was also exhibited by other phenolic substances; however, phenol caused a marked inhibition only at 10^{-1}M, a concentration at which no Q_{O_2} was observed. The addition of a dihydric phenol, sodium gentisate, also caused less inhibition than salicylate. The hypothesis of competition for available sulphate could not be substantiated, since addition of excess of carrier sulphate to the medium did not overcome the

Table 3. *Residual radioactive counts in hydrolysed, protein-free incubation medium, after precipitation of free ionic sulphate*

For conditions see incubation procedure (ii) under Methods.

	Counts/min./flask
Granulation tissue and *m*-aminophenol	2827
Granulation tissue alone	83
Liver slices and *m*-aminophenol	2601
Liver alone	58

inhibition caused by phenol or aminophenol. Inhibition in the presence of phenyl sulphate was considerably greater than that produced by similar concentrations of phenol. The effect could therefore not be attributed to hydrolysis of phenyl sulphate during incubation. Addition of carrier sulphate to the medium did not overcome inhibition of $^{35}SO_4^{2-}$ uptake by phenyl sulphate. It is possible that phenyl sulphate may be inhibitory by means of a competitive action on enzymes involved in sulphate metabolism. Of other sulphur-containing substances investigated, potassium methanesulphonate inhibited, whereas potassium ethyl sulphate failed to do so. The further elucidation of this problem and that of the mode of action of other inhibitors must await the availability of a satisfactory cell-free system.

The sulphation of phenol by granulation tissue was a somewhat surprising finding, since Segal (1955) found no such activity in a variety of other tissues. The only reported association of chondrosulphatase and aryl sulphatase activities has been found in the large periwinkle (*Littorea littorina*) by Dodgson & Spencer (1954). Our reasons for investigating the sulphation of phenol were, first, to study the specificity of the sulphating system and, secondly, to have a readily available substrate for future experiments on the problem of sulphation in granulation tissue. Hilz & Lipmann (1955) have shown the presence of a sulphate-activating and a transferring system in liver. It seems likely that a similar activating system occurs in granulation tissue and that a transfer to either carbohydrate or phenol may then take place.

SUMMARY

1. The Q_{O_2} and SO_4^{2-} uptake of guinea-pig granulation-tissue slices decreased with the maturation of the tissue after tendonectomy.

2. The SO_4^{2-} uptake by granulation tissue was stimulated by addition of glucose (2×10^{-2}M) or pyruvate (2×10^{-2}M), and was completely inhibited, together with Q_{O_2}, by fluoroacetate (2×10^{-2}M).

3. Sulphate uptake by the tissue was also inhibited by gentisate and phenol.

4. Phenol acted as an alternative substrate and was shown by chromatography to be converted into phenyl sulphate, which itself was an inhibitor of $^{35}SO_4^{2-}$ uptake by granulation tissue.

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The Metabolism of Sorbitol in the Human Subject

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Sorbitol has been used as a sweetening agent for diabetics since it was shown that moderate amounts of sorbitol taken by mouth by normal or diabetic subjects cause only a small and insignificant increase in the blood-sugar concentration (Thannhauser & Meyer, 1929). While this small increase was considered to be due partly to a slow rate of absorption from the intestine, it was also widely believed that, in man, orally ingested sorbitol is metabolized by a pathway not involving glucose (Payne, Lawrence & McCance, 1933).

Animal experiments have shown that as much as 64% of injected sorbitol may be excreted unchanged in the urine (Todd, Meyers & West, 1939; Smith, Finkelstein & Smith, 1940; Ellis & Krantz, 1941, 1943; Stetten & Stetten, 1951), whereas only about 3% of orally ingested material is lost in this way. Recently Stetten & Stetten (1951), Wick, Almen & Joseph (1951) and Wick, Morita & Barnet (1955) have injected uniformly labelled D-[^{14}C]sorbitol (D-[U-^{14}C]sorbitol) into normal and alloxan diabetic rats and recovered most of the radioactivity in the respiratory carbon dioxide. Hepatectomized rats were unable to metabolize sorbitol in this way. Embden & Griesbach (1914) and later Anschel (1930) suggested that sorbitol was metabolized via fruc-

tose, and sorbitol dehydrogenase, the enzyme effecting this conversion, was found in liver (Breusch, 1942). The properties of this enzyme have been further investigated by Blakley (1951). On the other hand, Wick *et al.* (1955) have shown that in alloxan-diabetic rats fed on a high fructose diet less fructose than sorbitol was oxidized to carbon dioxide, and suggest that at least two pathways exist for the oxidation of these two compounds.

There seemed need therefore for a re-investigation of the metabolism of sorbitol in man. Our initial experiments were with unlabelled sorbitol, and showed that after an oral dose of 35 g. of sorbitol given to either normal or diabetic subjects, sorbitol did not appear in the blood in concentrations greater than 2 or 3 mg./100 ml. Less than 2 % of the administered dose appeared in the urine, and less than 10 % in the faeces, even after the bacterial flora of the gut had been suppressed by aureomycin or terramycin. These last observations suggested that sorbitol was not significantly degraded in the intestine by bacteria but was metabolized by the human subject. The extent of this metabolism has therefore been investigated with D-[U-^{14}C]sorbitol. The activity of the expired carbon dioxide and that of the blood glucose have been measured after the ingestion of the labelled sorbitol as well as after a similar dose of uniformly labelled D-[^{14}C]glucose (D-[U-^{14}C]glucose). The results show conclusively that more than 70 % of orally administered sorbitol is metabolized to carbon dioxide by both normal and diabetic human subjects and that at least part of the sorbitol is converted into glucose. A brief report of this work has already been published (Adcock & Gray, 1956).

EXPERIMENTAL

Clinical material and methods

Experiments with unlabelled sorbitol. The subjects were six normal and eight diabetic adults. There were equal numbers of men and women in normal and diabetic groups. The diabetic subjects were all ambulatory patients attending the Diabetic Clinic of King's College Hospital; their diabetes was well controlled without insulin by diet alone. After the subject had fasted overnight, the bladder was emptied and capillary blood was collected from the lobe of the ear for glucose and sorbitol estimations. Sorbitol (35 g.) dissolved in 300 ml. of water was taken by mouth. Blood for analysis was collected at half-hourly intervals for 2·5 hr. In some cases urine was collected for 24 hr., and faeces for 3 days, after beginning the experiment.

Experiments with ^{14}C-labelled sorbitol. Labelled sorbitol was given by mouth to four subjects: *A* was a normal male, 28 years old; *B* and *C* were female and male diabetic subjects respectively, 75 and 70 years old respectively, neither of whom was receiving insulin; subject *D* was a female diabetic of 39 years, well controlled on 6 units/day of Lente insulin which, however, was not given on the day of the investigation.

Each subject fasted overnight, and then 35 g. of D-[U-^{14}C]sorbitol (total activity 30 μc) dissolved in 250–300 ml. of water was drunk over a period of 3 min. Normal diet was allowed 3 hr. after the sorbitol had been taken. At intervals during the following 24 hr. samples of expired air were collected into a Douglas bag for 5 min. periods, while the subject was sitting comfortably relaxed in a chair. Urine was collected at convenient intervals during the 24 hr. after the beginning of the experiment; faeces were collected in 24 hr. batches for 3 days. Three 30–40 ml. samples of blood for isolation and estimation of the radioactivity of the blood glucose were collected from subjects *C* and *D* by venepuncture from the antecubital vein at convenient times during the first 9 hr. after the sorbitol was given.

The investigations were repeated on subjects *C* and *D* with D-[U-^{14}C]glucose instead of the labelled sorbitol.

Methods

Estimation of sorbitol. Sorbitol in blood, urine and faeces was estimated by the method of Adcock (in preparation).

Estimation of blood sugar. Blood sugar (i.e. total reducing substances after removal of proteins) was estimated by the method of Nelson (1944) after precipitation of the protein with zinc sulphate and barium hydroxide (Somogyi, 1945).

Estimation of fructose. Fructose was estimated in blood plasma after protein precipitation by the Somogyi method, by the method of S. W. Cole (cited by Bacon & Bell, 1948).

Treatment of breath samples. No samples of expired air were collected from subject *B*. Small but unmeasured portions of each 5 min. collection from subject *C* and subject *B* after sorbitol were passed through an absorption tower containing CO_2-free N-NaOH. In the experiment on subject *C* after glucose, and in the two experiments on subject *D*, a measured portion of each 5 min. collection was drawn off over mercury from the delivery tube to the Douglas bag into a sample tube before passing through the absorption tower. In each case the total volume of expired air was measured by means of a gas meter.

Isolation of glucosazone from blood samples. The blood samples were collected with fluoride and oxalate as anticoagulants and treated with the Somogyi protein-precipitating reagents. After centrifuging the protein and after acidifying with acetic acid to prevent alkaline decomposition of the glucose, the supernatant was evaporated to dryness. The residue was extracted with ethanol, the filtered extract evaporated to dryness, and the residue taken up in 2 ml. of water. This solution was heated at 100° for 30 min. with 0·3 g. of crystalline sodium acetate and 0·2 g. of phenylhydrazine hydrochloride. Glucosazone readily crystallized and after cooling to room temperature was separated by centrifuging, washed by suspension in ethanol, filtered and rewashed with ethanol on a sintered-glass microfilter funnel. The m.p. was 205°, undepressed by admixture with authentic glucosazone. Weighed quantities of glucosazone were oxidized by wet combustion to CO_2 as described below.

Treatment of urine and faeces for radioactivity measurements. Portions of urine (2 ml. each) were evaporated to dryness after the addition of 0·1 ml. of NH_3 soln. (sp.gr. 0·88) and oxidized as described below to CO_2 for absorption in CO_2-free NaOH.

The complete faecal collections were weighed and homogenized in an Atomix blender after the addition of suitable known volumes of water. Small weighed amounts were

Table 1. *Total radioactivity in urine, faeces and breath after* 35 *g.* (30 μC) *of* D-[U-^{14}C]*sorbitol*

	Urine (0–24 hr. after sorbitol)		Faeces (0–3 days after sorbitol)		Breath (0–24 hr. after sorbitol)		Total radioactivity recovered	
	μC	% of dose	μC	% of dose	μC	% of dose	μC	% of dose
Subject *A*. Normal	0·45	1·5	3·94	13·1	24	80	28·39	95
Subject *B*. Mild diabetic	0·71	2·4	1·88	6·3	—	—	—	—
Subject *C*. Mild diabetic	0·8	2·7	0·28	0·9	26	87	27·08	90
Subject *D*. Severe diabetic	2·71	9·0	1·46	4·9	19·6	65	26·77*	90

* Diarrhoea after 2 hr.: loss estimated as 3 μC.

dried on a water bath before combustion to CO_2. When samples had been delayed before delivery to the laboratory the samples were dried after the addition of small quantities of alkali to avoid possible loss of volatile materials.

Combustion procedure. The various samples were oxidized with a chromic–sulphuric acid mixture (Van Slyke, 1954), the carbon dioxide being isolated as barium carbonate by the method of Berlin, Tolbert & Lawrence (1951) with minor modifications (Adcock, in preparation).

Radioactive materials. D-[U-^{14}C]Glucose and D-[U-^{14}C]-sorbitol were purchased from the Radiochemical Centre, Amersham.

Radioactivity measurements. All measurements of radioactivity were on barium carbonate mounted at infinite thickness on aluminium planchets of 15 mm. diam., with an end-window Geiger–Müller counter. The counts were corrected for background and were calibrated with a standard barium carbonate sample of high and known activity prepared by combustion of a small quantity of the labelled sorbitol solution given to the patients.

RESULTS

Experiments with unlabelled sorbitol. Of the six normal subjects, four were investigated in the fasting state and the other two at least 3 hr. after food. In neither group of patients did sorbitol given by mouth have a significant effect on the blood sugar. In all eight diabetic subjects, however, the oral administration of 35 g. of sorbitol was followed by a significant increase in the blood-sugar concentration ranging from 9 to 49 mg./100 ml. In neither normal nor diabetic groups did the concentration of sorbitol attain measurable levels in the blood; the method used was capable of estimating sorbitol in blood in concentrations greater than 2–3 mg./100 ml. In one normal subject who received 50 g. of sorbitol, sorbitol was present in a concentration of 9 mg./100 ml. after 1 hr.

The excretion of sorbitol in the urine during the 24 hr. after the sorbitol was given varied between 0·07 and 0·91 g., so that the loss in the urine amounted to less than 3 % of the dose administered. The majority of the excreted sorbitol was eliminated during the first 5 hr.; none could be detected after 24 hr. No unchanged sorbitol could be detected in the faeces of three subjects and only 10 % or less of the dose could be found in the faeces of those other patients whose gastro-intestinal tract had been sterilized by the adequate administration of antibiotics.

Fig. 1. Radioactivity of expelled CO_2 after 35 g. (30 μC) of D-[U-^{14}C]sorbitol. The specific activity of the CO_2/hr. has been calculated on the assumption that the output of CO_2 was constant at 4 g./5 min. (see text). ×, Subject *A*, normal; ▲, subject *C*, mild diabetic; ●, subject *D*, diabetic requiring insulin.

Table 2. *Total blood sugar and specific activity of blood glucose after* 35 *g. of* D-[U-^{14}C]*sorbitol of activity* 2·17 μC/*g. of carbon*

	Time after sorbitol (hr.)	Blood sugar (mg./100 ml.)	Specific activity of blood glucose (μC/g. of carbon)
Subject *C*	2·75	100–160	0·35
	4·25		0·22
	7·5		0·094
Subject *D*	1·0	228	0·49
	3·25	238	0·38
	9·25	279	0·058

Experiments with D-[U-^{14}C]*sorbitol.* Table 1 shows the total radioactivity in the urine, faeces and breath after ingestion of the labelled sorbitol. The total radioactivity of the urine from the normal subject and of the two mild diabetics was less than 3 % of the dose given, thus confirming the experiments

Table 3. *Total radioactivity in urine, faeces and breath after 35 g. (30 μc) of labelled glucose*

	Urine (0–24 hr. after glucose)		Faeces (0–3 days after glucose)		Breath (0–24 hr. after glucose)		Total radioactivity recovered	
	μc	% of dose	μc	% of dose	μc	% of dose	μc	% of dose
Subject *C*	1·63	5·4	2·08	6·9	22·7	76	26·41	88
Subject *D*	12·26	40·9	0·80	2·7	13·2	44	26·26	88

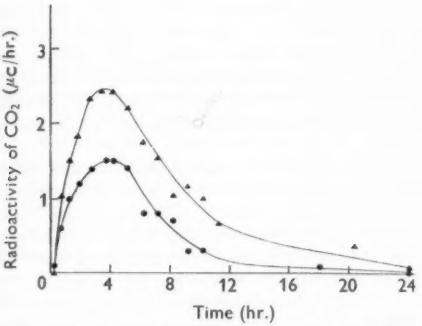

Fig. 2. Radioactivity of expelled CO_2 after 35 g. (30 μc) of D-[U-^{14}C]glucose. The specific activity of the CO_2/hr. has been calculated on the assumption that the output of CO_2 was constant at 4 g./5 min. (see text). ▲, Subject *C*, mild diabetic; ●, subject *D*, diabetic requiring insulin.

Table 4. *Total blood sugar and specific activity of blood glucose after 35 g. of* D-[U-^{14}C]*glucose of activity 2·17 μc/g. of carbon*

	Time after glucose (hr.)	Blood sugar (mg./100 ml.)	Specific activity of blood glucose (μc/g. of carbon)
Subject *C*	1·0	233	1·0
	4·0	156	0·64
	7·5	157	0·18
Subject *D*	1·0	396	0·96
	4·5	307	0·28
	7·75	315	0·14

with unlabelled material. Neither of these diabetic patients was excreting significant amounts of glucose in the urine. The radioactivity of the urine from the severe diabetic amounted to more than 9% of the dose given, presumably owing to the presence of labelled glucose. In the four subjects the radioactivity of the faeces passed during the 3 days accounted for between 1 and 13% of the labelled sorbitol given. The radioactivity of the faeces after 3 days was negligible.

In the normal subject and subject *C* (the mild diabetic from whom breath samples were collected) 80% of the administered radioactivity could be accounted for in the breath. In subject *D*, the diabetic who required insulin, only 65% could be accounted for in the breath, but the increased loss in the urine made up for this so that in all subjects investigated only 10% or less of the radioactivity could not be accounted for.

Fig. 1 shows that the radioactivity of the expired carbon dioxide was maximal between 3 and 4 hr. after the sorbitol feeding. In the normal subject the activity had declined to little more than 25% of the maximum value by 8 hr.; in the mild diabetic, the maximum activity was nearly equal to that observed in the normal subject and there was a decrease to 25% of that maximum within about 12 hr. The maximum radioactivity in the breath of the more severe diabetic was only about 50% of that observed in the other two subjects. In no subject was radioactivity detected in the breath after about 24 hr. Curves of the cumulative excretion of radioactivity in the breath did not show significant differences between the three subjects.

The specific activity of the blood sugar was determined at convenient intervals by isolation of glucosazone from venous blood in subjects *C* and *D* (Table 2). In the two experiments taken together, the activity appeared maximal within 1 hr. of the sorbitol ingestion and within 7–9 hr. declined rapidly to reach a value of 20% of the maximum observed. The sugar content of the specimens from subject *C* was inadvertently not estimated but this subject's blood sugar was known to be well maintained between 100 and 160 mg./100 ml.

Experiments with D-[U-^{14}C]*glucose.* Table 3 shows the total radioactivity in the urine, faeces and breath after ingestion of the labelled glucose. The total radioactivity of the urine from subject *C* was only 5% of the dose given. The radioactivity of the urine from subject *D*, the more severe diabetic, amounted to 41% of that given. The radioactivity of the faeces passed during the 3 days after the feeding of labelled glucose amounted to 6·9 and 2·7% of the dose given.

In the mild diabetic 76% of the administered radioactivity could be accounted for in the breath. In the more severe diabetic only 44% could be accounted for in the breath, but the increased loss in the urine made up for this so that in the two subjects only 12% of the radioactivity could not be accounted for.

Fig. 2 shows that whereas the radioactivity of the breath in the more severe diabetic followed a curve

almost the same as was found after sorbitol, in the mild diabetic the maximum activity attained was not so high as in the experiment with sorbitol. The peak of radioactivity in the severe diabetic, however, was flattened, presumably because of the excretion of labelled glucose, so that the total activity accounted for in the breath was of the same order after glucose as that found after sorbitol.

Table 4 shows that when D-[U-^{14}C]glucose was fed to subjects *C* and *D*, the specific activity of the blood glucose reached a higher level after 1 hr. than was found after the labelled sorbitol feeding. In both cases the activity at 7–8 hr. was only about 15% of that found at 1 hr.

The amount of fructose in blood samples obtained after sorbitol had been fed to subject *D* was found to be below the limit of sensitivity of the method, nor were significant amounts of fructose found in the blood of two other mild diabetic subjects who had taken unlabelled sorbitol. The method used would have determined concentrations of fructose above 2–3 mg./100 ml.

DISCUSSION

Absorption of sorbitol. The experiments with unlabelled sorbitol confirmed the absence of a significant increase in the blood-sugar concentration after a moderate dose (35 g.) of sorbitol, and that even in a diabetic subject the increase in blood-sugar concentration was less than 50 mg./100 ml., whereas increases above this were found in diabetic patients after a similar quantity of glucose. The absence of detectable sorbitol in the faeces suggested the possibility that bacterial degradation of sorbitol in the gut might be significant, but the experiments on subjects in whom antibiotic therapy had suppressed bacterial growth in the gut excluded this. The small, but significant, radioactivity in the faeces after the labelled material suggested that intestinal bacteria had in fact assimilated, and perhaps metabolized, a small proportion of both glucose and sorbitol. It therefore seemed likely that sorbitol was absorbed and that the failure of detectable amounts to appear in the blood was due to rapid metabolism, excretion, or storage in the body. Sorbitol is stated to be excreted by glomerular filtration (Smith *et al.* 1940) and a comparison of blood and urine concentrations is in agreement with this. The urine analyses, however, show that only a small quantity of orally ingested sorbitol was lost by renal excretion.

Metabolism of sorbitol to carbon dioxide. The measurements of breath $^{14}CO_2$ were discontinuous, and although the carbon dioxide content was reasonably constant at about 4% (v/v) the volume of expired air/5 min. varied according to the activity of the subject throughout the experiment. The expired ^{14}C has therefore been calculated on the assumption that the output of carbon dioxide was constant at 4 g./5 min. This corresponds well with the average daily carbon dioxide production for a sedentary worker calculated from the calorie requirement/hr., an R.Q. of 0·82 and a calorific value for oxygen of 4·825 kcal./l. (Hawk, Oser & Summerson, 1947). This was also in agreement with the known composition of the diet of the diabetic patients. The total radioactivity excreted in the breath has therefore been obtained by measuring the area from 0 to 24 hr. below the interpolated curves shown in Figs. 1 and 2. The results with labelled sorbitol clearly show that in the normal and in the mildly diabetic subject at least 75% of orally given sorbitol is rapidly metabolized to carbon dioxide. In the more severely diabetic subject the recovery of labelled carbon in the respiratory carbon dioxide amounted to only 65% and this lower figure was clearly due to the increased proportion of labelled material lost in the urine. The number of experiments is too small to assess the significance of the delay in the elimination of labelled carbon dioxide in subject *C*, the mild diabetic.

Possible pathway of metabolism of sorbitol. Blakley (1951) has shown that liver slices are capable of converting sorbitol into fructose and that the pathway for its metabolism is probably as follows:

Sorbitol $\rightleftharpoons$ fructose $\rightarrow$ fructose 6-phosphate
$\rightleftharpoons$ glucose 6-phosphate $\rightarrow$ glucose.

Sorbitol may be metabolized in the liver, may be stored as glycogen, or secreted into the blood as fructose or glucose and utilized elsewhere. The high specific activity of the glucosazones prepared from the blood filtrate must be attributed to either labelled fructose or labelled glucose or both in the blood. The highest observed activities of the glucosazones corresponded to 0·35 and 0·49 μc/g. of carbon, whereas the specific activity of the administered sorbitol was 2·17 μc/g. of carbon. Had the activity of the glucosazone been due to blood fructose formed directly from the labelled sorbitol, and therefore with the same specific activity as the sorbitol, between 16 and 23% of the total blood sugar would have been present as fructose. This would have amounted to 16–23 mg. of fructose/100 ml. in subject *C*, whose blood sugar was between 100 and 160 mg./100 ml., and 52 mg. of fructose/100 ml. in subject *D*. If there was isotopic dilution with unlabelled endogenous intermediaries, the blood fructose would have needed to be even higher. The demonstration that blood fructose cannot have exceeded 2 mg./100 ml. showed clearly that the radioactivity of the blood sugar was mostly, if not entirely, due to that of the glucose. Oral sorbitol is therefore presumably converted in the liver of the

human subject into fructose, a proportion of which is converted into glucose, which may in part be temporarily stored as glycogen and ultimately passed into the blood stream as glucose. The proportion of fructose not converted into glucose may be directly metabolized by the glycolytic pathway via fructose 6-phosphate.

The height of the peaks of activity of the respiratory carbon dioxide appeared to be inversely related to the blood-sugar concentrations. This would be expected because the higher the blood sugar the greater the blood-sugar pool with which the isotope was diluted, and also because loss of glucose in the urine occurred when the blood-sugar concentration was above the renal threshold, i.e. 180 mg./100 ml.

The relative specific activity of blood glucose and of respiratory carbon dioxide at corresponding times are compared in Table 5. As expected, the specific activity of the blood glucose after D-[U-^{14}C]sorbitol was lower than that after D-[U-^{14}C]glucose. After sorbitol, there was isotope dilution with pools of intermediaries in its conversion into glucose, as well as with the pools of glucose itself. After the labelled glucose, however, dilution occurred with the glucose pools only.

The peak of labelling of the carbon dioxide was lower and later than that of the blood glucose, which suggests that the labelled carbon dioxide was derived from the labelled glucose in the blood. Although the plasma bicarbonate was not measured in the subjects, and it may safely be assumed to have been not significantly altered, the system is very complex with its pools of bicarbonate, glucose and non-glucose precursors of carbon dioxide. For these reasons the fact that the maximum labelling of the breath $^{14}CO_2$ after sorbitol was greater than that after glucose cannot by itself be taken as an indication that sorbitol was in part oxidized without first being converted into blood glucose. But comparison of the ratios (peak ^{14}C in breath carbon dioxide)/(peak ^{14}C in blood glucose) after sorbitol and after glucose respectively should give information on this question, provided that the respective peaks come at about the same time in any pair of experiments. It is difficult to fix the blood-glucose peak from the three values available in each experiment, but owing to the speed of absorption one can be certain that the peak of labelling occurs within the first hour. Whatever curves are drawn, peaks of blood-sugar ^{14}C are found at about 0·5–1 hr. Table 6 shows the ratio (peak ^{14}C in carbon dioxide)/(peak ^{14}C in blood glucose) obtained by assuming that the 1 hr. values for the latter are maximal and, for subject *C* after sorbitol, by interpolating the results at 1 hr. These ratios are much higher after sorbitol than after glucose in both subjects *C* and *D* and show a significantly lower dilution of $^{14}CO_2$ relative to the blood-sugar ^{14}C from labelled sorbitol than from labelled glucose and provide definite evidence of an alternative pathway of metabolism of sorbitol to carbon dioxide.

Table 5. *Specific activity of blood glucose and expired* CO_2 *after* D-[U-^{14}C]*sorbitol or glucose, each of activity* 2·17 μc/*g. of carbon*

The specific activity of blood glucose was determined on glucosazones prepared from blood samples at the times stated. The specific activity of the CO_2 was determined from the curves presented in Figs. 2 and 3.

	Mild diabetic (*C*)			Severe diabetic (*D*)		
		μc/g. of carbon			μc/g. of carbon	
	Time (hr.)	Glucose	CO_2	Time (hr.)	Glucose	CO_2
Sorbitol experiment	2·75	0·345	0·145	1	0·49	0·073
	4·25	0·22	0·27	3·25	0·38	0·126
	7·5	0·094	0·168	9·25	0·058	0·055
Glucose experiment	1	1·0	0·099	1	0·96	0·065
	4	0·64	0·185	4·5	0·28	0·116
	7·5	0·18	0·115	7·75	0·14	0·053

Table 6. *Comparison of peak activities of* ^{14}C *in breath* CO_2 *and blood glucose*

	After glucose			After sorbitol		
	Peak ^{14}C (μc/g. of carbon)			Peak ^{14}C (μc/g. of carbon)		
	Breath CO_2 (*a*)	Blood glucose (*b*)	Approx. ratio (*a*/*b*)	Breath CO_2 (*a*)	Blood glucose (*b*)	Approx. ratio (*a*/*b*)
Subject *C*	0·19	1·0	0·2	0·28	0·6*	0·5
Subject *D*	0·12	0·96	0·1	0·15	0·49	0·3

* Interpolated value.

SUMMARY

1. Human diabetic and non-diabetic subjects excreted in the urine less than 3% of an oral dose of 35 g. of sorbitol. No sorbitol could be detected in the faeces.

2. After 35 g. of oral sorbitol, there was no significant increase in blood sugar in normal subjects, but in diabetic subjects the blood sugar increased by a much smaller amount than after glucose. The concentrations of sorbitol in the blood were immeasurably small.

3. Small and insignificant amounts of sorbitol were present in the faeces after sorbitol was fed to patients on antibiotic therapy.

4. Experiments with uniformly labelled D-[^{14}C]-sorbitol and D-[^{14}C]glucose showed that in the normal and in the mildly diabetic subject at least 75% of orally given sorbitol is rapidly metabolized to carbon dioxide. In a diabetic subject requiring insulin the recovery of labelled carbon in the respiratory carbon dioxide amounted to only 65% and this lower figure was clearly due to the increased proportion of labelled material lost in the urine.

5. It was shown that a substantial proportion of oral sorbitol is rapidly converted into blood sugar and the pathway of this conversion is discussed.

We thank Mrs F. Sharples for her advice on the radioactivity estimations, Dr R. D. Lawrence and the staff of the Diabetic Clinic at King's College Hospital for their help with the investigations on their patients, our three patients and one normal subject for their willing co-operation, and Dr J. J. Scott for much helpful discussion and advice. We are also indebted to Howards of Ilford Ltd. and Frank Cooper Ltd. for financial support of this investigation and to the former for seconding one of us (L. H. A.) to the Department of Chemical Pathology for the whole period of this work. Equipment purchased by the Joint Research Committee of King's College Hospital and Medical School was used for the radioactivity measurements

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The Use of Radioactive Isotopes in Immunological Investigations

10. THE FATE OF SOME INTRAVENOUSLY INJECTED NATIVE PROTEINS IN NORMAL AND IMMUNE RABBITS*

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Several workers have studied the elimination from the blood stream of antigenic proteins injected intravenously into normal or specifically immunized animals. The subsequent fate of these antigens after elimination from the blood has also been the subject of a number of investigations. However, none of these studies has yielded any detailed information about the rates of elimination of antigens from the blood of immune animals during the first hour or so after injection, or of the immediate fate of these eliminated antigens, though it is during this period that any reaction with circulating antibody would be expected to occur. Such studies might therefore be expected to throw some light on the behaviour of antibodies *in vivo*, and to yield information on their role in the defence mechanisms of the body.

The proteins that have been used in most previous studies on their fate in animals have been labelled in different ways by the introduction of comparatively

* Part 9: Francis, Mulligan & Wormall (1955*a*).

large numbers of readily detectable groups not normally present in the protein molecule. These methods of labelling proteins, however, usually result in considerable modification of their properties, so that they behave differently from native proteins.

The artificial introduction of radioactive isotopes is a convenient labelling method for experiments of this type since the introduction of small amounts of isotopic material will result in the production of 'trace-labelled' proteins whose properties are practically identical with those of native proteins. In addition, radioactive isotopes can be used to label proteins which have undergone extensive chemical modification. The whole subject of labelled proteins has been comprehensively reviewed by Coons (1954).

Friedberg, Walter & Haurowitz (1955) have shown that measurements of radioactivity in tissues 9 days or more after the injection of antigenic proteins trace-labelled with ^{131}I are not reliable as estimates of the amounts of the injected proteins actually present, which may be 40–70 times that indicated by the ^{131}I measurement. This, however, does not necessarily invalidate results based on differences between normal and immune animals obtained in a relatively short period after the injection.

This paper describes experiments in which native proteins, trace-labelled with ^{131}I, were injected intravenously into normal and immune rabbits, and the persistence of these proteins in the blood stream was determined in detail during the first hour or two after injection. In addition, many of the rabbits were killed 1–2 hr. after injection, and the antigen content of certain tissues was determined. A preliminary account of part of this work has already been given (Almond, Francis, Hawkins & Wormall, 1954).

MATERIALS AND METHODS

Horse-serum γ-globulins. These were prepared from horse serum by Kekwick's (1940) method for the separation of human-serum γ-globulins.

Bovine-plasma albumin. A 30% solution of bovine albumin (Armour Laboratories) was suitably diluted with 0·9% (w/v) NaCl solution.

Isotopic labelling of antigens. In the earlier experiments (with γ-globulins) the proteins were labelled with ^{131}I by the introduction of about 4 atoms of iodine/molecule of protein, with the I_2 in KI method of Francis, Mulligan & Wormall (1951). In later experiments (with bovine albumin) the modified method of Gilmore, Robbins & Reid (1954), as described by Francis, Mulligan & Wormall (1955*b*), was used. In all cases the labelled proteins were freed from uncombined iodine by dialysis against 0·9% NaCl solution in Visking-brand transparent cellulose tubing. The amount of ^{131}I used in each preparation was calculated so that each rabbit received an injection of antigen containing about 20 μc of ^{131}I and giving an initial count rate of approximately 1000/min./ml. of whole blood in the apparatus used for radioactivity measurements.

Radioactivity measurements. Panax E.H.T. and scaler units (types 44 or 100C) were used throughout. For measurements of ^{131}I in the blood, measured samples of 1 ml. or less of blood were haemolysed with 10 ml. of 0·02 N-NaOH and their radioactivity was measured in a Veall-type counter for liquid samples (Veall, 1948) (20th Century Electronics, type M6, M6H or M6M). Determinations of ^{131}I in tissues were made by a slight modication of the method of Perlman, Chaikoff & Morton (1941), the solution containing iodide being made up to a suitable volume and radioactivity measurements made directly on a part of this solution, in a Veall-type counter, instead of on a precipitate of silver iodide. In later experiments, tissues were homogenized in an MSE Ato-Mix Blender, and the radioactivity of samples of the homogenates was measured directly in a scintillation counter of the type described by Banks, Tupper, Watts & Wormall (1955).

Immunization. Most of the rabbits were immunized by the method of Proom (1943). Intramuscular injections of alum-precipitated antigen were given at about 14-day intervals, and good antisera were usually obtained 10–14 days after the second injection. In a few experiments with bovine albumin, rabbits were immunized by a course of intravenous injections of protein solution (3 mg. of N/kg. body wt.) at intervals of 7 days.

Qualitative and quantitative precipitin tests were carried out as described by Francis *et al.* (1955*b*).

Conduct of animal experiments. Adult rabbits (2·5–4 kg.) were injected with labelled antigen via the marginal vein of the right ear. Blood samples were obtained from the marginal vein of the left ear and collected in 1 in. diameter glass tubes in which one or two drops of saturated potassium oxalate solution had been evaporated. In many cases, after the injection of antigen into immune rabbits, varying degrees of anaphylactic shock were observed. Among other symptoms was a pronounced decrease in the peripheral circulation, rendering it difficult or impossible to collect any blood until the animal had recovered. This was largely overcome, in all the later experiments, by giving an intramuscular injection of an antihistamine (mepyramine maleate, 1·5 mg./kg. body wt.) about an hour before the antigen injection. Control experiments in normal and immune animals indicated that the antihistamine had no observable effect on the antigen elimination rate. The collection of blood samples was timed with a stop watch which had been started at the mid-point of the antigen injection; the times at the beginning and end of each bleeding were noted, and in calculating the results all the blood was assumed to have been taken at a time exactly half-way between these two times. Collection of the first blood sample was usually started not sooner than 2·5 min. after the injection, since preliminary experiments had shown that about 2 min. was necessary for the complete mixing of the injected antigen with the circulating blood.

Determination of blood volume. In some experiments the blood volumes of the rabbits were determined by the dye method (Courtice, 1943), modified by the use of a small 'loading' dose of 0·3 ml. of 0·2% solution of the dye (Evans Blue) given about 5 min. before the main dose. This was found necessary to saturate some extravascular receptors which rapidly remove a small amount of the dye from the circulation during the first few minutes after its injection.

Calculation of results. Throughout this work it has been assumed that the amount of radioactivity in the blood at any time is a direct measure of the amount of the injected antigen there (cf. Knox & Endicott, 1950). This may not be strictly true, for even within the first 2 hr. after injection, especially in immune rabbits, there may be a small amount of non-protein-bound iodine liberated into the blood stream as a result of de-iodination of the labelled antigen in the liver and other tissues (cf. Friedberg *et al.* 1955). However, the errors introduced by not making allowance for this breakdown are very small, and can reasonably be neglected in the experiments reported here.

The amounts of the injected antigen found in 1 ml. of each blood sample were plotted on a logarithmic scale against the times at which the samples were obtained. The points so obtained fell either on straight lines or on curves of the general shape shown by the continuous line in Fig. 1, which become straight after an initial period of up to 30 min. duration.

To analyse graphs of this second type, the equation for the line that fits best to the points on the straight section was calculated by the method of least squares, and this line was drawn on the graph and extrapolated to zero time. The ordinate values on this extrapolated line at the times corresponding to the points on the curved section of the graph were then subtracted from the experimentally determined ordinates of these points, and these differences were also plotted against the corresponding times. This second set of points was usually found to fall on a straight line, and the equation for the line best fitting these points was again calculated by the method of least squares, and the line drawn on the graph and extrapolated to zero time. The ordinate of a point on the experimental curve at any given time is thus the sum of the ordinates of the points on the two straight-line components at that time, indicating that the experimentally observed elimination rate is due to two independent concurrent processes each proceeding with an exponentially decreasing velocity. The half-lives of these two processes may be read off from the graphs.

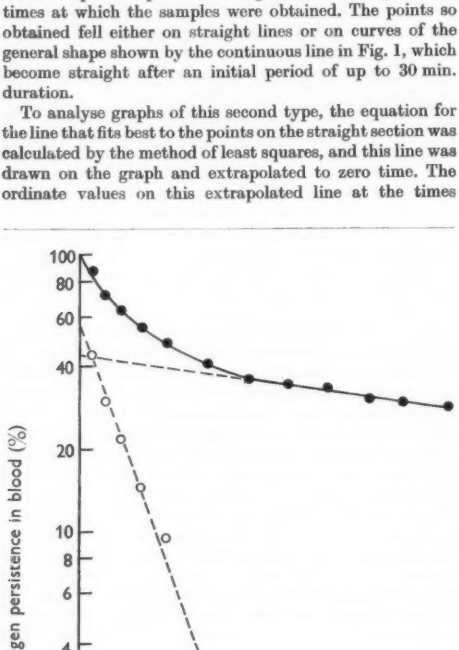

Fig. 1. Graphical analysis of typical curve of antigen persistence in the blood of a specifically immunized rabbit after the intravenous injection of antigen. (Antigen: ^{131}I-trace-labelled bovine albumin. Dose: 1 mg. of N/kg. body wt. Continuous line: experimental curve; broken lines: 'fast' and 'slow' components.)

The sum of the ordinates on the two component curves at zero time gives the total amount of injected antigen which would be present in each millilitre of blood immediately after the injection, assuming it to have mixed instantaneously with the whole of the circulating blood. Since the total amount of antigen injected is known, the animal's total circulating blood volume can therefore be calculated. The values calculated in this way were used in preference to those determined by the dye method when there was any discrepancy, e.g. when the turbidity of the serum rendered the dye method unreliable.

After the theoretical antigen concentration at zero time was calculated, either from the experimentally determined blood volume or by extrapolation of the component lines to zero time, all experimentally determined blood-antigen concentrations were expressed as a percentage of the initial concentration, and the curves redrawn. Fig. 1 shows the final curve in a typical experiment, the continuous line representing the experimentally determined elimination rate and the interrupted lines the two component processes. For convenience, the process which is virtually complete in 30 min. is referred to as the 'fast reaction', and the second process as the 'slow reaction'. The intercepts on the ordinate of the two lines at zero time also give the percentages of the total antigen present which are being subjected to elimination by the two processes separately.

Antigen in tissues. The amount of antigen found in any tissue was undoubtedly due in part to entrained blood, since no steps were taken to remove this blood in case this should also result in removal of any loosely bound antigen deposited in the tissue. Garvey & Campbell (1954) have shown that perfusion of the tissues washes out antigen deposited in liver or lungs, but the experiments recorded here were mostly carried out before this paper became available. In order to obtain an indication of whether antigen found in the tissues was entirely or only in part due to entrained blood, the results of radioactivity measurements on tissues are expressed as 'apparent blood volumes', i.e. antigen/g. of tissue divided by antigen/ml. of whole blood at the time of death. Evidently, if there is no deposition of antigen in the tissue, this ratio is the true blood volume (ml. of blood/g. of tissue) of the tissue. A comparison of the observed ratios in normal and immune rabbits should enable a decision to be made whether there has been any significant deposition of the antigen in the tissue of the immune rabbit.

In a few cases where animals received small doses of antigens which were almost completely eliminated from the blood by the time they were killed, the radioactivity of the tissues could be taken as a fairly accurate estimate of the

amount of antigen actually deposited, since the blood present in the tissues could have made only a negligible contribution to the total radioactivity.

RESULTS

Horse γ-globulins were chosen as the antigens to commence these studies since they are readily obtainable in a reasonably pure state by a simple method, and produce a good antibody response in rabbits. However, horse γ-globulins appear to behave as antigens in an abnormal manner in precipitin reactions *in vitro* (Francis, Mulligan & Wormall, 1954). They are not quantitatively precipitated by antibody, except possibly in regions of very large antibody excess, and in regions of antigen excess they are precipitated in amounts which are excessive relative to the amount of antibody in the precipitate. Since it appeared likely that they might also behave *in vivo* in a manner atypical of simple protein antigens, later experiments were carried out with bovine albumin.

Effect of dose level on persistence of protein antigens in circulating blood

The types of antigen-persistence curves obtained when normal and immune rabbits are injected intravenously with horse γ-globulin at various dose levels are shown in Figs. 2 and 3. The two immune animals injected at the higher rates (12·7 and 9·4 mg. of nitrogen/kg.) had approximately equal serum-antibody titres, but these were considerably higher than usual. In general, rapid elimination of approximately 50 % of the injected antigen occurred when injections were given at the rate of 1 mg. of antigen nitrogen/kg. body wt. The absolute amounts of antigen lost from the circulation in the first hour by these two rabbits were quite comparable (11·9 and 9·4 mg. of nitrogen), but the percentages were markedly different (35 and 58 % respectively). In the third rabbit, which received a much smaller dose, elimination was virtually complete. Normal rabbits in every case showed a much smaller percentage elimination in the first hour, and this percentage was dependent to a much smaller degree on the amount of antigen injected.

When bovine albumin was used as the antigen it was necessary to carry out a rough calibration of the antiserum before calculating the appropriate dose for injection. In a preliminary experiment 2 drops of the antiserum from each of four rabbits immunized with bovine-plasma albumin were treated with 5 drops each of serial dilutions of the antigen, and the amount of antigen required for maximal precipitation (which corresponds approximately to the equivalence zone) was judged by visual inspection. These rabbits were then injected with appropriate amounts of antigen to correspond to (i) moderate antibody excess, (ii) slight antibody excess, (iii) slight antigen excess and (iv) moderate antigen excess. In all but the fourth rabbit nearly the whole of the antigen was eliminated from the circulation within 1 hr., but in the fourth only about 50 % was so eliminated.

In order to be able to analyse the curves satisfactorily it is essential that a significant percentage of the injected antigen should be removed, but sufficient should remain to render the measurements reasonably accurate in the later periods. Since the precipitation of horse γ-globulin by antibody never, in practice, approaches 100 %, this condition could nearly always be realized by injecting 1 mg. of antigen nitrogen/kg. body wt. into rabbits immunized against this protein, but a rough calibration of the serum was always carried out before

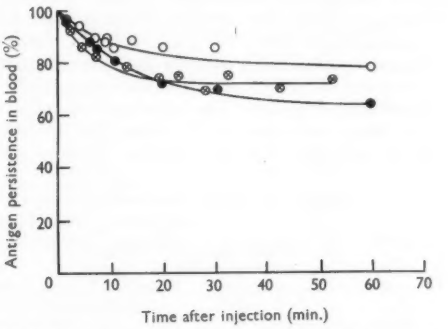

Fig. 2. Persistence of various doses of intravenously injected trace-labelled horse γ-globulin in the blood of normal rabbits. ○, 5 mg. of N/g. body wt.; ●, 1 mg. of N/kg. body wt.; ⊗, 0·33 mg. of N/kg. body wt.

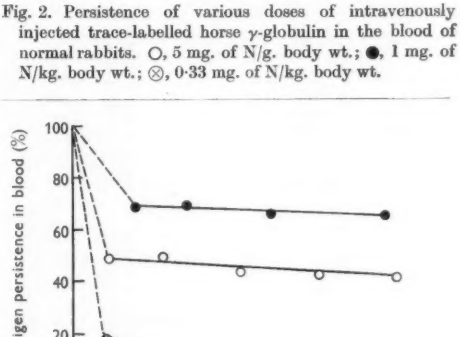

Fig. 3. Persistence of various doses of intravenously injected trace-labelled horse γ-globulin in the blood of rabbits immunized against horse γ-globulin. ●, 12·7 mg. of N/kg. body wt.; ○, 6·8 mg. of N/kg. body wt.; ⊗, 0·33 mg. of N/kg. body wt.

injecting rabbits immunized with bovine albumin, and the dose calculated to correspond to moderate antigen excess. Normal rabbits injected with bovine albumin gave, in nearly every case, straight-line persistence curves when plotted semilogarithmically.

Analysis of antigen-persistence curves

The antigen-persistence curves for a series of rabbits immunized by intramuscular injections of alum-precipitated bovine albumin, and injected with the trace-labelled antigen at varying intervals after the last immunizing injection, were analysed. Normal rabbits were also included in the group to act as controls.

The amounts of antigen injected necessarily varied with individual immune animals, and in the control rabbits injections of comparable size were given. The results (Table 1) show that the average half-lives of the slow reaction in the normal and immune groups were the same, but the reaction appears to have been slightly faster in immune rabbits tested a relatively short time after the last immunizing injection. The fast reaction, on the other hand, was observed in all immune animals, but in only two out of ten controls. In about one-half of the immune rabbits of this series the velocity of the fast reaction did not correspond to an exponential function, i.e. the calculated curve was not a straight line but a smooth curve. It was not possible, however, to analyse this curve further to establish whether it consisted of two or more exponential processes. The percentage of injected antigen removed by the fast reaction in these cases is therefore determined by difference, and no half-life can be given. The significance of this finding will be discussed more fully elsewhere (Francis & Hawkins, in preparation). In a series of experiments carried out with horse γ-globulin as the antigen essentially similar results were obtained, but a relatively small percentage of the antigen was also eliminated by a fast reaction in normal rabbits (9–25 % as compared with 26–67 % in the immune rabbits). In both normal and immune rabbits the graphs of the fast reaction corresponded with simple exponential functions having similar half-life values to those observed in the first series.

In neither series did there appear to be any relationship between the time an animal had been in the immune state and either the half-life of the fast reaction or the percentage of antigen eliminated by this reaction.

Table 1. *Analysis of persistence curves of* ^{131}I *in the blood of normal and immune rabbits after the injection of labelled bovine-plasma albumin*

Time after last immunizing injection (days)	Antigen injected (mg. of N/kg. body wt.)	^{131}I removed by fast reaction* (%)	Half-life of fast reaction (min.)	Half-life of slow reaction (min.)
		Normal rabbits		
—	0·09	11	3·7	265
—	0·09	0	—	275
—	0·16	0	—	269
—	0·16	0	—	226
—	0·29	0	—	309
—	0·30	0	—	310
—	0·37	0	—	209
—	0·91	10	6·5	326
—	1·19	0	—	199
—	2·20	0	—	245
			Mean value	263
		Immune rabbits		
8	3·04	43	4·6	182
8	2·90	33	3·4	129
14	0·64	(55)	—	144
15	1·97	39	7·9	268
15	2·39	38	5·0	203
15	0·60	22	11·3	147
15	1·20	(36)	—	282
15	1·17	(61)	—	285
22	2·58	(72)	—	277
22	1·35	(55)	—	300
22	1·62	(48)	—	450
133	0·11	76	8·2	350
133	0·10	67	10·1	420
			Mean value	264

* Figures shown in parentheses were observed in rabbits in which the curve representing the fast reaction is complex; hence its half-life cannot be calculated. The figures are equal to that percentage of the ^{131}I which is not removed from the blood by the slow reaction.

Table 2. *'Apparent blood volumes' of tissues of normal and immune rabbits killed 1 hr. after the intravenous injection of labelled horse γ-globulin*

For definition of 'apparent blood volume' see text (Materials and Methods).

Antigen injected (mg. of N/kg. body wt.)	5·03	0·37	1·06	5·82	1·04	
	Normal rabbits					
Tissue						Mean
Liver	0·16	0·16	0·18	0·18	0·068	0·15
Lungs	0·16	0·24	0·24	0·24	0·21	0·22
Spleen	0·19	0·19	0·23	0·21	0·14	0·19
Heart	0·11	0·023	—	—	—	0·07
Skeletal muscle	0·018	0·049	—	—	—	0·03
Antigen injected (mg. of N/kg. body wt.)	12·6	6·79	5·84	1·07	0·57	0·50
	Immune rabbits					
Tissue						
Liver	0·19	0·43	0·41	0·32	0·85	2·1
Lungs	0·71	—	0·36	0·32	0·28	0·36
Spleen	0·34	0·53	0·20	0·46	0·40	0·69
Heart	0·12	0·13	—	—	—	0·055
Skeletal muscle	0·014	0·013	—	—	—	0·038

Elimination of soluble antibody–antigen complexes

Bovine albumin trace-labelled with ^{131}I was added to a calibrated rabbit antiserum of known antibody titre to give a very small precipitate in the region of high antigen excess. After incubation at 37° for 1 hr. the mixture was kept at 0–4° for 112 hr. to ensure complete precipitation of the insoluble antibody–antigen complexes, and centrifuged at 0°. The clear supernatant was injected into four normal rabbits, and the persistence of the labelled antigen determined. Each rabbit received 6·4 mg. of antibody nitrogen (0·24 μmole of antibody) and 48 mg. of antigen nitrogen (4·26 μmoles of antigen). If it is assumed that antibodies are bivalent when they form soluble complexes in the antigen excess zone (Marrack, Hoch & Johns, 1951) then 0·48 μmole (5·44 mg. of nitrogen, 11·3 %) of the antigen should have been in the form of these complexes. The remainder must have been present in the free state. The persistence curves were all simple exponential ones and had half-lives ranging from 167 to 242 min. (mean 197 min.).

Radioactivity measurements on cells and plasma

Blood samples from normal and immune rabbits injected with horse γ-globulin, taken at intervals from 5 to 60 min. after the injections, were centrifuged and the cells washed twice with 0·9 % NaCl solution. Radioactivity measurements were then made simultaneously on the whole blood and on measured volumes of the washed and haemolysed cells. The results showed that a significant amount of radioactivity was centrifuged down with the cell fraction in the immune rabbits immediately after the injection (up to 25 % during the first 10 min.) but that this diminished rapidly with time (1–2 % after 1 hr.). By contrast, in normal rabbits only an insignificant amount of the radioactivity could be found in the cell fraction at any time. Similar results were obtained in rabbits injected with bovine albumin.

Table 3. *'Apparent blood volumes' of tissues of normal and immunized rabbits killed 60–90 min. after the intravenous injection of labelled bovine-plasma albumin*

For definition of 'apparent blood volume' see text (Materials and Methods).

Organ	No. of rabbits	Apparent blood volume: Range	Apparent blood volume: Mean
	Normal rabbits		
Liver	10	0·10–0·24	0·16
Lungs	10	0·18–0·41	0·27
Spleen	9	0·10–0·23	0·15
Kidneys	6	0·12–0·34	0·21
	Immunized rabbits, tested 2–14 days after immunization, but showing no immune response		
Liver	12	0·12–0·26	0·18
Lungs	13	0·17–0·40	0·28
Spleen	11	0·12–0·19	0·14
Kidneys	9	0·14–0·32	0·22
	Individual values of 'apparent blood volumes' in immunized rabbits showing an immune response		
Liver	0·95	0·72	2·0	6·7
Lungs	0·57	0·31	1·0	9·8
Spleen	0·83	—	—	4·7
Kidneys	0·45	0·21	0·69	—

These results suggest that a precipitin reaction analogous to the familiar precipitin reaction *in vitro* may be occurring in the blood stream. If this is so, the precipitate must be rapidly removed from the blood, presumably by phagocytosis.

Deposition of antigens in tissues

A number of the animals used in the antigen persistence measurements were killed 1–2 hr. after the injection, and blood and tissue samples were removed for radioactivity determinations. The results are given in Tables 2 and 3. The apparent blood volumes of the various organs of different normal rabbits are reasonably constant for each organ irrespective both of the dose injected and the nature of the antigen. The figures for heart and skeletal muscle in the immune animals are not significantly different from those for the corresponding tissues in normal animals, but the apparent blood volumes of the livers, lungs and spleens of the immune rabbits show much greater individual variation, and are, with few exceptions, considerably higher than those of the corresponding organs of the normal rabbits. Thus there seems no doubt that the liver, lungs and spleen play some part in the removal of intravenously injected antigen from the circulation of immune rabbits.

In order to obtain a more quantitative picture of the part played by these organs, some immune animals were injected with very small doses of the antigens, so that only about 10% of the injected antigen was still circulating when the animals were killed 90 min. after the injection. The blood contained in the tissues could only have contributed very slightly to their total radioactivity, thus the actual amounts of antigen deposited in the liver, lungs and spleen could be determined fairly precisely. The results, expressed as the percentage of the injected dose present in the whole of each tissue, are shown in Table 4 and indicate that the liver is quantitatively the most important organ in removing antigen from the blood stream. This is mainly on account of its large weight compared with the other organs examined, for the concentration of antigen/g. of tissue is not invariably highest in the liver. The percentage of the injected antigen accounted for in the blood and the three organs examined is quite high, but several organs (e.g. lymph nodes, thymus and bone marrow) which may contribute to the removal of antigen have not been examined in these experiments. The lymph and tissue fluid must also have contained significant amounts of the antigen.

Development of the immune response after injection of bovine albumin

The course of the development of the primary immune response in rabbits after an immunizing injection of antigen has been studied by Knox & Endicott (1950), and by Talmage, Dixon, Bukantz & Dammin (1951). They investigated the disappearance of bovine albumin and bovine γ-globulin from the blood of normal rabbits after a single intravenous injection, and showed that antibodies first appeared in the plasma about 9 days after the injection of albumin (12 mg. of nitrogen/kg. body wt.) and about 7 days after the injection of the globulin (6 mg. of nitrogen/kg. body wt.). Just before the antibodies were first detectable a marked acceleration of the rate of disappearance of the injected antigen was observed, which was interpreted as an early manifestation of the immune response. A different approach has been used in this work to study the same phenomenon.

Rabbits were given a single intravenous injection of non-radioactive bovine albumin (3 mg. of nitrogen/kg. body wt.) and then a test injection of ^{131}I-labelled antigen (0·3 mg. of nitrogen/kg. body wt.) 2, 5, 9 or 14 days later. The amount of the test injection was deliberately kept small, so that if the immune response had developed it would be readily detectable, and a considerable proportion of the injected antigen would be rapidly removed from the blood. None of these animals appeared to have any circulating precipitating antibody at the time of the test injection, as judged by qualitative precipitin tests, though it is possible that there may have been small traces which this test was not sensitive enough to detect. All the rabbits tested 2, 5 and 9 days after the immunizing injection failed to give the characteristic immune response of rapid elimination of part of the antigen from the blood stream during the first hour after injection, and the apparent blood volumes of their livers, lungs and spleens fell within the range usually encountered in

Table 4. ^{131}I *Content of tissues of immune rabbits killed* 90 *min. after the injection of small doses of labelled antigen*

	^{131}I content (% of injected dose)				
Antigen injected ...	Horse γ-globulin			Bovine-plasma albumin	
Tissue					
Liver	65	53	59	66	71
Lungs	5·9	4·1	17·5	8·4	2·3
Spleen	1·0	1·2	0·7	0·3	0·3
Blood	15*	10*	5*	4·4*	7·7*
Total recovery	86·9	68·3	82·2	79·1	81·3

* Calculated from the estimated blood volume.

normal animals (see Table 3). Three out of six animals tested 14 days after immunization showed a rapid elimination of about one-half of the injected test dose, and their organs had the high apparent blood volumes characteristic of immune animals. The other three animals in this group behaved in a manner indistinguishable from normal animals.

A later attempt to reproduce this phenomenon in six rabbits tested 14 days after the immunizing injection was inconclusive. Only one animal showed an immune response after the test injection. The serum of this animal did contain a very small amount of antibody, which was demonstrated by a more sensitive precipitin test than that used for testing the sera of the first set of animals.

These results suggest that the immune response first develops about 14 days after the intravenous injection of bovine albumin in amounts of 3 mg. of nitrogen/kg. body wt. Knox & Endicott (1950) showed that circulating antibodies to bovine albumin first appeared about 9 days after an intravenous injection of this protein. The reason for the difference between their results and ours is not apparent, but it might be due to the larger doses of antigen injected by these workers.

DISCUSSION

Talmage *et al.* (1951) have shown that 99% of an injected protein could be eliminated from the blood of immune rabbits in 24 hr., but the observations reported here demonstrate that, provided only small amounts of antigen are injected, over 90% may be removed from the circulation in less than 1 hr. by a 'fast reaction' having a half-life of approximately 10 min. It seems evident from the behaviour of the animals injected with different amounts of antigen (e.g., Fig. 2) that there is an upper limit to the amount of antigen which an individual rabbit is capable of removing by this fast reaction, and that this is determined by the amount of antibody available to react with it at the time of injection. The presence of small amounts of antigen in the cell fraction when the blood is centrifuged also supports the hypothesis that an *in vivo* precipitin reaction is associated with the mechanism of the fast reaction. The alternative explanation that the antigen may have been taken up by the blood cells does not seem very probable in view of the rate at which it leaves the circulation, though it is possible that some of the leucocytes may have acted in this way since they are believed to contain appreciable amounts of antibody. There was, however, no evidence of a fast reaction when soluble antibody–antigen complexes were injected into normal rabbits, which strongly suggests that the formation of a discrete precipitate is an essential part of this process.

The rapidity with which the precipitated antibody–antigen complex is completely removed from the vascular system remains, however, a most striking phenomenon.

When larger amounts of antigen are injected, the fast reaction is still evident, and although a smaller percentage of the injected protein is eliminated within the first hour, the amount removed by the fast reaction may still be determined by graphical analysis, either by reading off the intercept on the ordinate at time zero of the fast reaction component of the curve, or by subtracting from 100% the corresponding intercept of the slow component.

The fast reaction was also observed in all normal rabbits injected with horse γ-globulin, though only rarely with bovine albumin. We have frequently observed that γ-globulins are far more liable than albumins to undergo partial denaturation during chemical treatment, and it appears probable that some part of these 'more damaged' proteins, even though in a number of cases they were Seitz-filtered before injection, may be eliminated from the blood stream of normal animals by a phagocytic mechanism similar to that responsible for the removal of particulate foreign matter (cf. Francis & Hawkins, 1957). McFarlane (1956), in studies on the elimination of injected labelled plasma proteins over a period of several days, has also observed that proteins trace-labelled with ^{131}I may in some cases be eliminated from the circulation of normal rabbits in a manner different from that of biologically labelled [^{14}C]proteins, owing to a more rapid removal of part of the [^{131}I]protein, but this discrepancy disappeared if the [^{131}I]protein was first 'filtered' in the circulation of one animal before injection into another. This effect was also more marked with [^{131}I]serum globulin than with [^{131}I]serum albumin, and in our experiments also the occurrence of the fast reaction in normal rabbits injected with bovine albumin was exceptional.

The rapid rate of removal of injected antigens from the blood of immune rabbits is comparable with the rate of removal of injected particulate material such as colloidal chromic phosphate. Dobson & Jones (1952) have shown that this is removed from the blood of mice with a half-life of only 30 sec. This is certainly a faster reaction than is observed after antigen injection, but the mechanism for eliminating the two types of substance may be the same. The rate of elimination of particulate antibody–antigen complexes from the blood will be determined by at least three factors: the rate of mixing of antigen with the plasma so that it can react with all the antibody present, the rate of formation of the antibody–antigen precipitate and the rate of removal of that precipitate. Complete mixing of the antigen with the circulating plasma probably takes up to 2 min.: the rate of flocculation

of the antigen–antibody complexes in these particular systems cannot be assessed with any certainty, but it is likely to be of the order of a few minutes (cf. Boyd, 1947), so that one of these two factors is almost certainly the rate-limiting reaction. Thus it is quite likely that these insoluble antigen–antibody complexes are eliminated from the blood by the same mechanisms which remove other particulate material.

Native antigenic proteins are also eliminated from the blood of normal (non-immune) rabbits, but at a considerably slower rate. This loss of antigen from the blood is due to the diffusion of the antigen into the protein space of the animals, and it has been studied in more detail by Knox & Endicott (1950), Talmage *et al.* (1951), and Cohen, Holloway, Matthews & McFarlane (1956). The extravascular diffusion is usually complete in 24–48 hr. and is succeeded by an even slower loss of antigen from the blood, which is interpreted as being due to its metabolic breakdown. This last phase of antigen disappearance from the blood is not apparent in the short-term experiments reported here, but the loss of antigen by diffusion is constantly observed and in immune rabbits it is superimposed upon the more rapid elimination of the antigen from the blood by the fast reaction.

There is some suggestion from the experimental results (Table 1) that the half-life of the slow reaction in immune animals shortly after their last immunizing injection may be less than that observed in normal animals. It is not certain whether this difference is significant, but it might be expected that immune animals would have a slightly greater elimination rate during this second phase since (*a*) the concentration gradient of the antigen between plasma and lymph would be greater in the immune animals owing to removal of the antigen in the lymph by reaction with antibody there, and (*b*) any continued liberation of antibody into the blood stream would contribute to a more rapid elimination rate. In the series of animals injected with bovine albumin the half-life of the slow reaction does, in fact, show some tendency to increase when the animals have been in the immune state for a long time and are therefore unlikely to be still actively synthesizing specific antibody.

The possibility that soluble antigen–antibody complexes might be removed from the blood at a different rate from that of the uncombined antigen has also been tested, but there seems to be very little difference in the elimination rates of these two kinds of substances.

The tracing of injected antigens into the tissues has confirmed the observations of Libby & Madison (1947), Ingraham (1951), Laws & Wright (1952) and of Garvey & Campbell (1954), that the livers, lungs and spleens of immune animals take up antigen, but has shown that this phenomenon occurs more rapidly than was demonstrated by these workers. When only small amounts of antigen are injected into immune rabbits, and almost all the antigen is removed from the blood in the first hour after injection, the total amount of antigen found in the liver, lungs and spleen in nearly all cases is only a little below that injected. It therefore seems probable that, though other organs undoubtedly play some part in the response of immune rabbits to antigen injection, these three organs are of major significance in this respect.

There are several ways in which antigens, injected intravenously into immune rabbits, could be deposited in the tissues. First, the antigen–antibody precipitates formed in the blood stream could either be filtered out directly by the capillaries or be transported to the tissues after phagocytosis by cellular elements in the blood. Secondly, they could leak out of the circulation into the tissues at places where hypersensitivity reactions have damaged the tissues (Garvey & Campbell, 1954). Thirdly, antigens could be taken up by antibodies which are bound to the tissues in some way. This third possibility will be discussed more fully elsewhere.

The first possibility was envisaged by Banks, Francis, Franklin & Wormall (1950) in their studies on the fate of lipovitellin injected into normal rabbits. This protein is precipitated by the low ionic strength of the plasma and is rapidly filtered out by the lungs. This factor might be expected to operate particularly in the lungs, for they contain the first capillary circulation that the intravenously injected antigen must traverse in its passage through the vascular system. Some form of filtration may also be occurring in the liver and spleen where the blood has to traverse sinuses containing large numbers of cells. Other particulate material is removed from the blood stream by filtration in the lungs, for Odeblad, Dobson, Odeblad & Jones (1955) have demonstrated that colloidal chromic phosphate forms small intra-capillary emboli in the lungs of mice after intravenous injection. However, in the elimination of antigens from the blood of immune animals it seems unlikely that this is the only mechanism operating, for there is no evidence that antigen is deposited in other tissues, such as skeletal muscle, which certainly have a capillary circulation.

The observations of Garvey & Campbell (1954) on the non-specific deposition of antigens in the lungs of immune animals are of importance in considering the possibility that antigens may be deposited in tissues because of hypersensitivity reactions. No attempt has been made to confirm these observations in the present work; it may be significant, however, that in the experiments described here,

antigens were still deposited in tissues when the animals had been protected from anaphylactic shock by injection of an antihistamine drug which would be expected to minimize, if not abolish, the hypersensitivity reactions which damage the tissues. This does suggest that even if antigens may be deposited in tissues non-specifically as a result of tissue damage, there are other means which are also operative.

SUMMARY

1. Horse-serum γ-globulins and bovine-plasma albumin, both trace-labelled with ^{131}I, have been injected intravenously into normal rabbits, and their persistence in the blood stream has been measured during the first 2 hr. after injection. A relatively small loss of the protein is observed during this period, and it has been shown that this loss is frequently brought about by two processes (the 'fast reaction' and the 'slow reaction'). A method for calculating the amounts of protein acted upon by each of these processes is described.

2. When the same proteins were injected into specifically immunized rabbits, a much larger proportion of the protein was removed from the blood by the fast reaction, probably owing to an *in vivo* precipitin reaction followed by a rapid phagocytosis of the precipitate.

3. The rapid removal of bovine-plasma albumin from the blood of immunized animals has occasionally been observed with rabbits which had received a single immunizing injection of the antigen 14 days previously, but this phenomenon was not observed 2, 5 and 9 days after the immunizing injection.

4. Soluble antibody–antigen complexes containing trace-labelled bovine-plasma albumin are removed from the blood of normal rabbits only slightly more rapidly than the antigen itself.

5. After the injection of antigens into immune rabbits, considerable deposition of antigen occurs in the liver, lungs and spleen. If such small amounts are injected that 80–90% of the injected antigen is removed from the blood in 1 hr., the recovery of ^{131}I in these organs is almost equal to that which has disappeared from the blood.

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The Use of Radioactive Isotopes in Immunological Investigations

11. THE FATE OF SOME CHEMICALLY MODIFIED PROTEIN ANTIGENS IN NORMAL AND IMMUNE RABBITS

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Proteins, chemically modified by the introduction of atoms or groups that are not normally present, have been widely used by immunochemists, particularly for studies on the specificity of antibodies (see Landsteiner, 1945). Modified proteins containing radioactive halogen or coloured azo groupings have also been used in a few studies of the fate of proteins after injection into animals, since their rate of elimination from the plasma can be followed by radioactivity or colorimetric measurements, and histological techniques can be used to trace them in tissues.

Sabin (1939) injected a coloured azoprotein into rabbits and detected dye granules in cells throughout the reticulo-endothelial system. When repeated injections of this protein were made, specific antibodies were produced, and it was observed that the amounts of dye found in the liver and spleen were inversely proportional to the antibody titres of the sera. Kruse & McMaster (1949) injected azoprotein containing a very highly coloured and very diffusible blue dye (*Echt-Saure-Blau*) into mice and observed that the dye was still detectable in their livers after 15 weeks.

The rates of elimination of coloured azoproteins from the blood of rabbits have been studied by Pratt & Gregersen (1941), and by Gitlin, Latta, Batchelor & Janeway (1951). The latter workers observed that the more highly labelled the protein was, the more rapidly did it disappear from the blood stream. Qualitatively similar observations were made by Fine & Seligman (1943), when they injected proteins labelled with varying amounts of ^{82}Br into dogs, and observed that the rates of elimination of these proteins were proportional to the number of atoms of halogen introduced. These findings suggested that these chemically modified proteins behaved differently from native proteins when injected into rabbits and other animals. This was confirmed directly by Dixon, Bukantz & Dammin (1951), who showed that a native protein (bovine γ-globulin), trace-labelled with ^{131}I, was eliminated from the blood of normal rabbits much more slowly than an azoprotein derived from it by coupling with diazotized *p*-aminobenzoic acid. The azoprotein also appeared to be retained longer than the native protein in a number of tissues (particularly the liver and spleen), and its breakdown products were excreted much more slowly than those derived from the native protein.

Because they have been so widely investigated in other connexions, it was decided to study the behaviour of these chemically modified proteins after intravenous injection into normal and specifically immunized rabbits, in continuation of the studies reported previously (Francis, Hawkins & Wormall, 1957). Since antibodies formed in response to the injection of one particular chemically modified protein (the homologous antigen) will react with other proteins into which the same determinant groups have been introduced (heterologous antigens), it was of interest to investigate the fate of both homologous and heterologous antigens in immune animals. Such a study is only possible if chemically modified proteins are used. Two types of proteins were selected: highly iodinated proteins, in which the principal determinant groups are the 3:5-diiodotyrosyl residues in the polypeptide chains (Wormall, 1930); and mustard-gas sulphone proteins (HO_2-proteins) produced by treating native proteins with an excess of 2-dichloroethyl sulphone (mustard gas sulphone, HO_2). The principal determinant groups of these proteins involve the 1:4-thiazan 1:1-dioxide structure (Boursnell, Francis & Wormall, 1946; Francis, Mulligan & Wormall, 1955*b*).

MATERIALS AND METHODS

Highly iodinated proteins (*iodoproteins*). Bovine-serum γ-globulins or rabbit-serum γ-globulins were iodinated as described by Banks, Francis, Mulligan & Wormall (1951), except that the concentration of added ammonia was reduced to 2N. For the preparation of radioactive iodoproteins a solution of 0·1 N-I_2 in 0·1 M-KI containing a suitable quantity of Na^{131}I was used. The products were freed from unbound iodine, iodide, etc., by dialysis rather than by precipitation.

HO_2-Proteins. These were prepared from either ovalbumin or bovine-serum γ-globulins as described by Francis, Mulligan & Wormall (1955*a*). Again, purification was effected by dialysis rather than by precipitation.

Radioactivity measurements. In blood and tissues ^{131}I was measured as described by Francis *et al.* (1957).

In blood and tissues ^{35}S was determined after conversion into $BaSO_4$ by the method of Bailey (1937). The $BaSO_4$ was centrifuged and transferred quantitatively to nickel planchets 2·5 cm. in diameter with the aid of a few drops of 50% ethanol. The dried precipitates were counted under a bell counter (G.E.C. type EHM2S). Corrections for self-absorption were made as described by Francis, Mulligan & Wormall (1954).

Protein-bound ^{131}I. Blood samples were haemolysed with 5–10 vol. of 0·01 M-KI, and the proteins precipitated by the slow addition, with stirring, of 1 vol. of 100% (w/v) trichloroacetic acid. The precipitated proteins were centrifuged, washed twice with 5 vol. of 10% trichloroacetic acid, dissolved in N-NaOH and made up to a convenient volume for radioactivity measurement.

Weighed samples of about 5 g. of liver homogenate were mixed directly with 5 ml. of 20% trichloroacetic acid, centrifuged and washed twice with 10 ml. of 10% trichloroacetic acid. Because of the difficulty of dissolving the precipitate, radioactivity measurements were made on the combined supernatants; the protein-bound ^{131}I was then determined by the difference between the ^{131}I in the whole homogenate and that in the trichloroacetic acid extract.

Immunization of rabbits and injection of radioactive antigens. These were performed as described by Francis *et al.* (1957).

Calculation of results. The percentages of radioactivity in the blood at various times after the injection of radioactive antigens were calculated as described by Francis *et al.* (1957), and graphs of the persistence of radioactivity in the blood were plotted. It is not possible to analyse these persistence curves mathematically in any simple way as can be done after the injection of native proteins into rabbits.

The amounts of radioactivity found in the tissues of the rabbits are expressed either directly, or as percentages of the amount injected, since the amount of radioactivity in the blood at the time of death was always so low that it did not contribute significantly to the amount in the tissue.

RESULTS

Iodinated proteins

All the immune animals used for these experiments were immunized with iodinated bovine γ-globulin, and at the time of the experiment had antisera which reacted strongly with the test antigen used. Three groups (three or four in each group) of immune animals were injected with iodinated rabbit γ-globulin (0·1, 0·3 and 0·95 mg. of nitrogen/kg. body wt. respectively), and one group with the homologous antigen (0·5 mg. of nitrogen/kg. body wt.). Four groups of normal rabbits were also injected similarly.

The curves (plotted on a linear scale) for the antigen persistence in the blood of individual rabbits in each group were in reasonable agreement, and there were no significant differences in the general shapes of the curves, or in the percentages of either antigen eliminated in the first 30 min. from the blood of control or immune rabbits. In all cases over 50% of the antigen was eliminated within 5 min., over 80% within 15 min. and 82–95% within 30 min. After reaching a minimum value by 30 min., in many cases the amount of ^{131}I in the blood commenced to rise again, as shown in the typical curve in Fig. 1. That this rise is probably due to the release of ^{131}I-containing fragments split off from the iodoproteins after they had been removed from the blood stream was shown by determinations (on two normal and two immune rabbits) of the amount of protein-bound ^{131}I 10–15 min. and 60 min. after the injection of radioactive protein. The non-protein-bound ^{131}I increased from less than 10% of the total radioactivity in the blood at 10 min. to over 30% at 60 min. with both normal and immune rabbits (Table 1), so that it seems unlikely that there is any marked difference in the rates of catabolism of iodoproteins in normal and immune rabbits, at least during the first hour after injection.

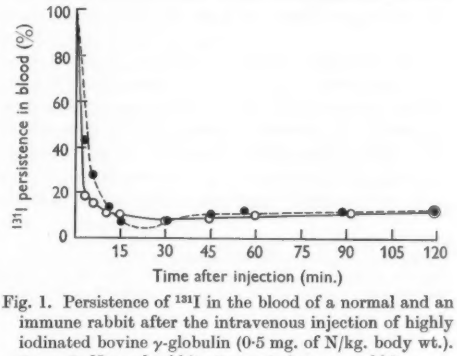

Fig. 1. Persistence of ^{131}I in the blood of a normal and an immune rabbit after the intravenous injection of highly iodinated bovine γ-globulin (0·5 mg. of N/kg. body wt.). ●– – –●, Normal rabbit; ○——○, immune rabbit.

Table 1. *Distribution of* ^{131}I *in blood of two normal and two immune rabbits after intravenous injection of highly iodinated rabbit* γ*-globulins* (0·3 *mg. of N/kg. body wt.*)

Time after injection (min.)		Normal (%)		Immune (%)	
10–14	Protein-bound ^{131}I	92*	91†	95*	94*
	Non-protein-bound ^{131}I	8*	9†	5*	6*
60	Protein-bound ^{131}I	62	58	69	66
	Non-protein-bound ^{131}I	38	42	31	34

* Samples of blood taken at 10 min. † Samples of blood taken at 14 min.

The results of ^{131}I determinations on tissues of four rabbits killed 1 hr. after injection are shown in Table 2. The immune rabbits had considerably more ^{131}I in their livers and lungs than had the normal ones, even though the total elimination of the injected antigen from their blood was slightly less. In all cases, well over one-half of the ^{131}I in the liver was protein-bound, i.e. it was mainly due to the antigen and not to its breakdown products. Thus the deposition of antigen in these organs may, in some circumstances, be more characteristic of the immune response than the rate of elimination of the injected antigen from the blood stream.

Table 2. ^{131}I *Content of tissues of two normal and two immune rabbits killed* 60 *min. after intravenous injection of highly iodinated rabbit* γ*-globulins* (0·3 *mg. of* N/*kg. body wt.*)

	Percentage of injected ^{131}I in tissue			
	Normal		Immune	
Tissue				
Liver	26·3 (70)*	27·3 (66)*	41·5 (80)*	43·5 (74)*
Lungs	0·5	0·3	11·4	8·9
Kidneys	3·2	3·3	3·1	2·8
Blood†	10·5	10·0	12·3	10·9

* Figures in parentheses give the percentage of liver ^{131}I which was protein-bound.

† Based on estimated blood volumes of 60 ml./kg. in each case.

Mustard gas sulphone-treated proteins (HO_2*-proteins*)

The rabbits used for these experiments were immunized with either HO_2-bovine γ-globulin, or with HO_2-ovalbumin, and the test injections were all made with the HO_2-γ-globulin (0·3 mg. of nitrogen/kg. body wt.), labelled with ^{35}S. Three of the four animals immunized with HO_2-γ-globulin had sera which reacted strongly with the test antigen, whereas the fourth showed only a weak reaction, but the two animals immunized with HO_2-ovalbumin had sera which reacted weakly with the homologous antigen and not at all with the test antigen.

The persistence of ^{35}S in the blood of some of these rabbits, for 60 min. after the injection, is shown in Fig. 2 (average curves for each group). The HO_2-protein is not removed from the blood of normal rabbits quite so rapidly as is a highly iodinated protein and there is no indication of the liberation of ^{35}S-containing fragments into the blood stream during the first hour after injection, since the blood radioactivity decreases progressively throughout this time. This label, therefore, seems a more

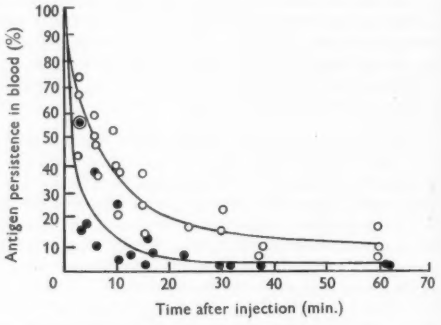

Fig. 2. Persistence of intravenously injected HO_2-bovine γ-globulin (0·3 mg. of N/kg. body wt.) in the blood of four normal rabbits and three immune rabbits. ○, Normal rabbits; ●, immune rabbits with sera reacting strongly with test antigen.

Table 3. *Antigen content of tissues of rabbits killed* 60 *min. after intravenous injection of 2-dichloroethyl sulphone-treated bovine* γ*-globulin*

Group *A*: two rabbits immunized with 'sulphone bovine γ-globulin'. Serum (1) gave a strong reaction with the test antigen, serum (2) a weak reaction. Group *B*: two rabbits immunized with 'sulphone ovalbumin'. Sera (1) and (2) reacted weakly with the homologous antigen, but gave no detectable reaction with the test antigen. Group *C*: two normal rabbits.

	Group *A*		Group *B*		Group *C*	
	(1)	(2)	(1)	(2)	(1)	(2)
Liver						
Concn. of antigen (μg. of N/g. of tissue)	6·6	11·0	6·8	6·1	3·9	5·4
Total antigen (%)	67	79	67	50	48	52
Lungs						
Concn. of antigen (μg. of N/g. of tissue)	0·69	1·8	0·94	0·27	0·23	0·21
Total antigen (%)	0·80	2·0	1·0	0·30	0·20	0·30
Blood						
Concn. of antigen (μg. of N/g. of tissue)	0·09	0·20	0·52	0·34	0·27	0·32*
Total antigen (%)†	1·7	4·0	10·2	6·8	5·4	6·3*

* Sample of blood at 30 min. The 60 min. sample, which would have had an antigen content only slightly lower, was lost.

† Based on estimated blood volumes of 60 ml./kg. in each case.

satisfactory one for chemically modified proteins, and HO_2-proteins are probably more suitable than highly iodinated proteins for studies *in vivo*.

The antigen was eliminated from the blood of the three highly immune rabbits considerably faster than from the blood of the normal rabbits, although its rate of elimination in the slightly immune rabbit injected with homologous antigen was not significantly different from that observed in the controls. The two rabbits immunized with HO_2-ovalbumin also behaved in a manner indistinguishable from the controls.

The results of analyses for ^{35}S in the lungs and livers of a small group of six rabbits are shown in Table 3.

As with the rabbits injected with iodoprotein, there was considerable deposition of the antigen in the liver and lungs of normal animals. The two animals immunized with HO_2-γ-globulin, however, showed a significantly greater deposition in these tissues, and this difference was actually more pronounced in the animal with the lower serum-antibody titre. Of the two animals immunized with HO_2-ovalbumin, whose sera gave no detectable reaction with the test antigen, only one showed any marked difference from the controls. In view of the differences in the amounts of antigen in the entrained blood in these tissues, calculations have also been made of the amounts of antigen actually deposited in the tissues, by making an allowance in each case for their blood content, based on the 'apparent blood volumes' of fifteen normal rabbits injected with ^{131}I-trace-labelled protein (Francis *et al.* 1957). These revised figures presented exactly the same overall picture as those given in Table 3, and they are therefore omitted from the table.

DISCUSSION

The fate of intravenously injected, chemically altered proteins differs considerably from that of native proteins (cf. Pratt & Gregersen, 1941; Dixon *et al.* 1951; Gitlin *et al.* 1951). In particular, they are eliminated very rapidly from the blood of normal animals (Haurowitz & Crampton, 1952), though the mechanism responsible for this behaviour is uncertain. It seems probable that it is connected with the extensive chemical alteration to the molecules which has occurred, accompanied by marked changes in their configuration. Crampton, Reller & Haurowitz (1953) have suggested that iodoproteins may form complexes with serum proteins which would rapidly be removed from the circulation.

The rate of elimination of these extensively modified proteins from the blood of specifically immunized animals during the first hour after injection is not necessarily greater than that shown by normal animals. In our experiments no significant differences could be observed in the elimination rates of iodoproteins by the two groups, but HO_2-proteins were eliminated significantly faster by immune animals having strong antisera at the time of injection.

The rate of deiodination of iodoproteins in the liver (and possibly other tissues) appears to be markedly greater than that of iodine trace-labelled proteins, but there is no evidence of any corresponding loss of the labelling group from HO_2-proteins in the short period studied. Iodoproteins therefore appear to be inferior to HO_2-proteins for investigations of this type *in vivo*.

Because of the large amounts of these antigens removed from the blood of normal rabbits, they are more extensively deposited in the livers than are native proteins. However, the recovery of radioactivity in the tissues examined is frequently rather low, for more than one-half of the injected antigen may not be accounted for. With the iodoproteins this may in part be due to catabolism of the antigen, followed by uptake of ^{131}I by the thyroid and excretion of non-protein-bound iodine through the kidneys. Certainly, the recovery of ^{131}I in the livers of normal animals injected with iodoprotein appears to be lower than the recovery of ^{35}S in the livers of normal animals injected with HO_2-protein, but in both groups it appears likely that there are considerable quantities of either the antigen or its breakdown products deposited in other tissues which were not examined.

More radioactivity is found in the livers and lungs of immune animals than in those of normal ones, even in circumstances where the antigen elimination rate from the blood is no faster than in normal animals. Thus the deposition of antigens in the liver and lungs seems to be a more constant feature of the immune response than their rapid elimination from the blood.

Proteins which have been extensively chemically modified may be regarded as more 'foreign' to the body than are native proteins from an immunologically distinct species, and it would appear that the bodily defence mechanisms against invasion of the blood stream by substances of this more 'foreign' nature are less dependent on a specific immune reaction than are the mechanisms concerned with substances of a less 'foreign' nature, even though the invading substances may be strongly antigenic.

SUMMARY

1. Highly iodinated γ-globulins and 2-dichloroethyl sulphone-treated γ-globulins are removed very rapidly from the blood of normal rabbits after intravenous injection. A considerable proportion of

the eliminated protein can be recovered in the liver but very little in the lungs.

2. Iodoproteins injected intravenously into specifically immunized rabbits are eliminated from the blood at the same rate as in normal animals, but immunized rabbits with good antisera to 2-dichloroethyl sulphone-treated proteins eliminate these proteins from their blood more rapidly than do normal animals.

3. With both antigens the deposition in the liver and lungs is greater in immune animals than in controls.

4. One hour after injection of iodoprotein at least one-third of the radioactivity in the blood is no longer protein-bound.

5. The deiodination of iodoproteins in the tissues renders them unreliable for studies *in vivo*, even for comparatively short-term experiments.

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The Formation of Hippuric Acid

THE INFLUENCE OF BENZOATE ADMINISTRATION ON TISSUE GLYCINE LEVELS

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The conjugation of benzoic acid with glycine has been extensively studied by many workers. One aspect of the problem that has received much attention is the origin of the glycine used for conjugation, but the exact source nevertheless remains uncertain. In the present investigation the problem has been approached by studying the concentration of certain tissue metabolites under various experimental conditions; this paper deals with the influence of the administration of benzoate upon the concentration of tissue glycine. The aim was to disturb the normal precursor → glycine reaction as little as possible, since the administration of relatively large amounts of suspected glycine precursors may cause unusual metabolic pathways to be followed. Few studies comparable with the present one appear to have been carried out. Christensen, Cooper, Johnson & Lynch (1947) and Vries & Alexander (1948) found that the administration of benzoate produced a fall in the concentration of free glycine in the blood of man, and Christensen, Streicher & Elbinger (1948) observed a similar decrease in the blood and liver of the guinea pig, although there was only a small change in the concentration of glycine in the kidney and no detectable change in muscle.

In the present investigation the influence of the administration of benzoate on the free glycine of various rat tissues and on the protein glycine of liver has been studied. Experiments in which blood glycine was investigated were also repeated on the rabbit since consecutive blood samples could be obtained from a single animal. In most of the experiments with the rabbit, urine samples were

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also collected in order to study the kinetics of the conjugation reaction with respect to the conjugated moiety (cf. Bray, Thorpe & White, 1951).

METHODS

Animals and diet. Female albino rats (body wt. usually 130–170 g.) and female rabbits (2·5–3·3 kg. body wt.) were used. The animals were maintained on a normal diet of cubes or pellets except that, to eliminate any effects of dietary glycine, they were restricted to a 'protein-free' diet during the 48 hr. before an experiment. This diet for rats consisted of wheat starch 90%, arachis oil 6·4% and salt mixture (Hubbell, Mendel & Wakeman, 1937) 3·6%, and that for rabbits contained wheat starch 89%, paper pulp 9% and salt mixture 2%; the starch and salt mixture were made into a smooth paste with water and the paper pulp was added and mixed with the paste. The mixture was baked at 100° until hard and then broken up into small lumps. These diets contained less than 1 mg. of N/g. A ration (10 g. for the rat and 50 g. for the rabbit) of the diet was given 48 and 16 hr. before the experiment began. Rats and rabbits were kept in wire cages with wide-mesh floors.

Dosage. Sodium benzoate was administered by intraperitoneal injection of an aqueous solution at a dose level of approximately 400 μmoles/100 g. body wt., unless otherwise stated. The volume injected was generally 1 ml. for a rat and 10 ml. for a rabbit.

Preparation of tissues for analysis

Blood. Samples were obtained from rats by cardiac puncture under ether anaesthesia; coagulation was prevented by collecting blood in a syringe rinsed with citrate and discharging it into 1% (w/v) sodium citrate in 0·9% (w/v) saline (0·2 ml./ml. of blood). In the rabbit, blood was collected from a marginal vein of the ear; coagulation was prevented either by the use of oxalate or, more frequently, by collecting the blood directly into tungstic acid mixture (equal parts of 0·66 N-H_2SO_4 and 10% (w/v) $Na_2WO_4,2H_2O$).

Other tissues. Rats were killed either by a sharp blow on the head or, if blood samples were required, by severing the aorta after cardiac puncture. The required organs were rapidly dissected out, washed with water, dried with filter paper and placed in tared beakers immersed in a freezing mixture of solid carbon dioxide and ethanol. All tissues were in the freezing mixture within 2 min. of the death of the animal. The individual tissues from a particular group of rats were pooled. The contents of each beaker were allowed to thaw slightly and then minced very finely with scissors and well mixed. A sample (usually 1·5–3 g.) was then frozen solid and kept in this condition until worked up (up to 5 hr.).

The sample, mixed with tungstate mixture (1·5–2 ml./g. of tissue) and 15–20 ml. of water, was comminuted in a macerator (Measuring and Scientific Equipment Ltd.) for 3–5 min. at 14 000 rev./min. (Histological examination of a kidney preparation, made under these conditions except that tungstate mixture was replaced by water, showed that no intact cells remained.) The suspension was diluted with water to 50 ml. and the protein removed by centrifuging. The tungstic acid filtrate was stored overnight in the refrigerator until assayed.

The protein fraction separated by centrifuging was suspended successively twice in 5% (w/v) trichloroacetic acid (10 ml.), twice in acetone (15 ml.) and once in ether (15 ml.); the suspension was centrifuged and the supernatant was discarded at each stage (cf. Winnick, Friedberg & Greenberg, 1948). The protein was air-dried at room temperature.

Analytical methods

Free glycine. The tungstic acid filtrate described above was assayed by the method of Alexander, Landwehr & Seligman (1945), as modified (modification A) by Christensen, Riggs & Ray (1951); this depends on the deamination of glycine by ninhydrin to form formaldehyde, which is determined with chromotropic acid (1:8-dihydroxynaphthalene-3:6-disulphonic acid). A Spekker photoelectric absorptiometer was used with Ilford 605 filters. Samples of filtrate containing 1·5–3 μg. of glycine/4 ml. were used.

The term 'free' glycine refers to that glycine which appears to be free when determined by customary analytical procedures.

Total non-protein glycine. This was determined in the hydrolysed tungstic acid filtrate. Traces of protein, if present, were first removed by acidification (0·1 ml. of 6 N-HCl/10 ml. of filtrate), heating for 1 min. in a boiling-water bath and filtering off the coagulated protein. A sample of the filtrate (5 ml.) was hydrolysed for 36 hr. with conc. HCl (2·5 ml.) in a boiling-water bath and then evaporated to dryness. Water was added to the residue and again removed by evaporation. Water (5 ml.) was added to this residue and a sample of the solution (4 ml. for blood, 1 ml. for tissues) assayed as above. Control experiments with blood and liver in which deproteinized filtrates were compared with diffusates prepared by a method similar to that of Hamilton & Archibald (1944) gave results agreeing within 0·2 mg. of glycine N/100 g. (cf. Christensen & Lynch, 1946).

Hippuric acid glycine. A sample of the tungstic acid filtrate (5 ml. for blood, 10 ml. for other tissues) was further acidified with 1 ml. of 2 N-H_2SO_4 and continuously extracted with redistilled peroxide-free ether for 6 hr. The solvent was removed from the extract and 6 N-HCl (2 ml.) was added to the residue, which was then refluxed on a sand bath for 2 hr. The acid was removed by evaporation to dryness. The residue was dissolved in 5 ml. of water and 3 ml. was taken for glycine assay.

Protein glycine. The air-dried protein fraction described above was hydrolysed by heating with 6 N-HCl (9 ml.) in a boiling-water bath for about 36 hr. The hydrolysate was diluted to 50 ml. with water and the 'humin' filtered off. The samples (0·1 ml.) used for assay did not contain sufficient HCl to alter significantly the pH of the buffer used in the glycine determination.

Peptide non-protein glycine. This term is used to express the difference between total non-protein glycine and free glycine plus, if the amount is significant, hippuric acid. This is probably mainly glutathione (Arnstein, 1954).

Analysis of urine

The rate of excretion of hippuric acid by the rat was determined on urine samples collected after the administration of benzoate. A correction was applied for the excretion under 'base-line' conditions determined under similar conditions on the previous day. To stimulate the flow of urine, the rats were given water (2·5–5 ml., depending on body wt.) by stomach tube at three equal intervals during the period of urine collection, which was usually for 4·5–5 hr. after benzoate administration. Conjugation of the

larger doses (about 400 μmoles/100 g. body wt.) used is not complete in this period. Hippuric acid was estimated by the method of Bray, Clowes, Thorpe, White & Wood (1952). In most experiments with rabbits, urine was collected as described by Bray *et al.* (1951) and the urinary ether-soluble acid (Bray, Neale & Thorpe, 1946), after correction for free benzoic acid, was taken as a measure of the excretion of hippuric acid.

Plan of experiments

Rats. In general, six groups of two rats of similar weight were used. Those in groups A and B, injected with water, served as controls and those in groups C, D, E and F were injected with sodium benzoate. The latter were killed at suitable time intervals after injection (up to 5 hr.). Rats in group A were killed at the same time as those in group C, and those in group B at the same time as those in group F. The mean values from the two control groups were taken as the zero-time values for each experiment. In some experiments, however, rats of a control group were killed at the same times as those in each experimental group.

Rabbits. Two blood samples were collected before the administration of benzoate and others at suitable intervals after administration. Control experiments showed that no significant variation in the concentration of free glycine occurred during the period of observation in the absence of benzoate. In most experiments urine was also collected.

RESULTS

Effect of administration of benzoate on the free-glycine level of blood and tissues

The concentration of free glycine in rat liver fell after the administration of benzoate and a steady low level about 50% of the initial level was established within 0·5–1·5 hr. of dosage (Table 1). More detailed results from two typical experiments are shown in Fig. 1. The relative magnitude of the change appeared to be independent of the size of the dose within the range (170–470 μmoles/100 g. body wt.) studied. The correlation coefficient, r, was 0·01, ($n = 23$). The steady low glycine level was of short duration in curve B of Fig. 1 on account of the very small dose level used; it was calculated that excretion of hippuric acid was complete in 4 hr. (curve F). In the experiments at higher dose levels excretion of hippuric acid was not complete until after about 8 hr.

Administration of benzoate to the rat produced a fall in blood free glycine, which, as shown in Table 1, appears to be significant. The relative fall (to about 80% of the original level) was smaller than in liver and was unrelated to dose level ($r = 0·09$, $n = 18$). Similar results were obtained when consecutive blood samples were taken from a

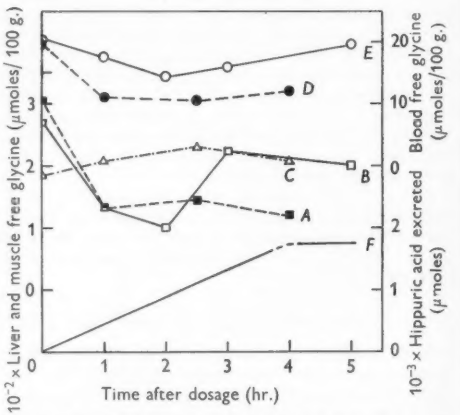

Fig. 1. Effect of intraperitoneal injection of benzoate upon the concentration of free glycine in rat liver, muscle and blood. Doses (given in parentheses) are in μmoles/100 g. body wt. *A* (■), Liver (350); *B* (□), liver (170); *C* (△), muscle (380); *D* (●), blood in Expt. A; *E* (○), blood in Expt. B; *F*, excretion curve for hippuric acid for Expt. B. *F* was calculated with $k_g = 0·08$ hr.$^{-1}$ and $v_h = 36$ μmoles/100 g. body wt./hr. (see Bray *et al.* 1952). Each experimental point represents the result of analysis of the pooled tissue from two rats killed at the time indicated.

Table 1. *Free-glycine concentration of rat tissues when steady low level has been attained after injection of sodium benzoate*

Mean dose level 400 μmoles/100 g. body wt. in 1 ml. of solution. Control rats were injected with water (1 ml.). Individual values were obtained from determinations on pooled material from two or three rats, except for blood, where some values were obtained from only one rat. Values are means ± S.D. with ranges in parentheses. P is the probability that there is no significant change in concentration after administration of benzoate.

	Control (g_1)			After injection of benzoate (g_2)			
Tissue	No. of expts.	Total no. of rats	(μmoles/100 g. of tissue)	No. of expts.	Total no. of rats	(μmoles/100 g. of tissue)	P
Liver	33	69	268 ± 42 (190–346)	21	45	144 ± 46 (88–252)	< 0·001
Blood	29	52	20 ± 4 (11–26)	22	38	16 ± 6 (11–33)	< 0·01
Kidney	6	14	582 ± 33 (540–614)	4	10	637 ± 119 (514–795)	> 0·1
Muscle	9	21	199 ± 33 (155–262)	10	23	215 ± 49 (164–342)	> 0·1
Intestine	3	9	158 ± 14 (144–169)	3	9	180 ± 16 (163–194)	> 0·1

Table 2. *Effect of administration of sodium benzoate on the concentration of free glycine in rabbit blood*

Doses were given by oral (Or) or intraperitoneal (Ip) routes.

Expt. no.	Days on protein-free diet before expt.	Dose level (μmoles/100 g. body wt.)	Route of injection	Blood free glycine (μmoles/100 ml.) Initial	Mean new level after dose	New level as % of initial level	Rate of excretion, v_h (μmoles/hr./100 g.)
1	0	420	Or	137	63	46	—
2	2	250	Ip	53	24	45	22
3	2	180*	Or	56	22	40	—
4	2	430	Ip	81	23	28	—
5	9	390	Or	116	33	28	24
6	9	370	Or	121	16	13	27

* Benzamide was administered instead of benzoate.

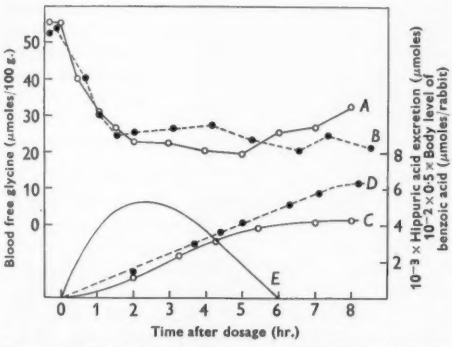

Fig. 2. Effect of administration of benzamide and benzoic acid upon the concentration of free glycine in rabbit blood. Doses (given in parentheses) are in μmoles/100 g. body wt. *A*, Blood free glycine (benzamide, 180); *B*, blood free glycine (benzoate, 250). The corresponding excretion of hippuric acid is shown in curves *C* and *D* respectively. Curve *E* shows the calculated body level of benzoate in the benzamide experiment (k_g, 0·08 hr.$^{-1}$; k_b, 0·32 hr.$^{-1}$; v_h, 750 μmoles/rabbit/hr.). Rabbits had been kept on a protein-free diet for 2 days before the experiment.

rabbit which had received benzoate by intraperitoneal injection (Fig. 2, *B*). The experiment was not continued long enough to determine whether the beginning of the return to the original free-glycine level coincided with completion of conjugation of the benzoate but Fig. 1 (*E* and *F*) and the results of the experiment with benzamide described below (Fig. 2, *A*) suggest that this is likely. The results from six experiments, including the one with benzamide, are shown in Table 2. The initial values for blood free glycine are all within the range found in rabbits which had had 2 days of 'protein-free' diet (53–157 μmoles/100 g. body wt. in 12 expts., mean 91 ± 30 S.D.). There appears to be no correlation between the extent of the fall in the level of blood free glycine and the dose administered (and, presumably, the body level of benzoic acid). The rate of conjugation of benzoic acid as judged by the rate of excretion of hippuric acid in three experiments is directly related to the percentage fall in the level of blood free glycine. While this is in the expected direction the significance of the difference in the rates of excretion observed is doubtful. The new level of blood free glycine took longer to establish (about 1·5–3 hr.) than in the rat (about 1 hr.). The conclusion that the fall in concentration is independent of the dose (body) level of benzoate is supported by the results of the experiment in which a small dose of benzamide was administered (Fig. 2, *A*, *C*, *E*). Benzamide forms benzoate relatively slowly *in vivo* (Bray *et al.* 1951), so that lower body levels of benzoate, which persist for a longer time, can be obtained by the administration of benzamide than by giving small doses of benzoate which are very rapidly eliminated. The fall in the level of blood free glycine was similar to that obtained with benzoate (Fig. 2, *B*) and independent of the body level of benzoate until very low body levels were reached, when the concentration of blood free glycine began to rise again. The establishment of a low level of blood free glycine and its independence of body level down to very low body levels of benzoate is to be expected from the fact that the conjugation reaction follows the kinetics of a zero-order reaction down to very low body levels of benzoate, when the kinetics change to those of a first-order reaction (Bray *et al.* 1951).

The transition period after dosing with benzoate was studied more closely in the rabbit. The concentration of blood free glycine fell approximately exponentially (Fig. 3) to reach the low constant level. The data of Christensen *et al.* (1947) and Vries & Alexander (1948) show that the free-glycine concentration of human plasma changes in the same way as that of rabbit blood after the administration of benzoate.

No significant change in the concentration of free glycine in rat muscle, kidney or small intestine was found after the administration of benzoate (Fig. 1, *C*; Table 1).

Effect of administration of benzoate on other forms of glycine in rat tissues

Preliminary results, summarized in Table 3, indicated that only insignificant changes in the concentration of peptide non-protein glycine occurred in the chosen rat tissues after the administration of benzoate. Since it is likely that the liver protein would be that which would be degraded if the glycine of protein were required for conjugation, the effect of the administration of benzoate upon the concentration of liver-protein glycine in the rat was investigated. The results showed that no significant change was produced by benzoate up to 4 hr. after injection. The mean control value (8 expts.) was 7560 ± 713 (S.D.) μmoles/100 g. of liver (316 ± 19 μmoles/100 g. body wt.), and the corresponding value after the administration of benzoate (9 expts.) was 7130 ± 927 (287 ± 33). If liver-protein glycine were the main source of glycine used for conjugation a fall in concentration in the liver should be detectable several hours after dosage, owing to the relatively low turnover rate of liver mixed protein; e.g. Shemin & Rittenberg (1944) reported that about 10% is replaced daily. Arnstein & Neuberger (1951) reported that they were unable to find a significant reduction in the total protein N of rat liver after the administration of benzoate.

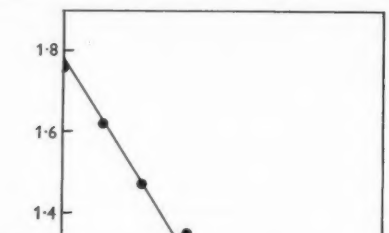

Fig. 3. Effect of intraperitoneal injection of benzoate (430 μmoles/100 g. body wt.) upon concentration of blood free glycine in the rabbit; g is the value for free glycine at a given time and g_2 that for the mean low level finally reached (23 μmoles/100 ml.).

Rate of conjugation of benzoate with glycine in the rat

The rate of excretion of hippuric acid in the rat seems to be approximately equal to the rate of conjugation of benzoate with glycine, since little hippuric acid could be detected in the tissues, except kidney, after the administration of benzoate. The rate of excretion of hippuric acid was approximately constant (cf. rabbit, Bray *et al.* 1951). The mean value from six experiments was 36 ± 6 (S.D.) μmoles/100 g. body wt./hr. (range 24–43). The rate did not appear to vary markedly with body weights of 112–194 g. These values were obtained with rats dosed with water, but control experiments showed that the rate of urine flow did not have a marked effect upon the rate of excretion of hippuric acid. Sheppeck & Griffith (1936) were also unable to find any relation between the rate of excretion of hippuric acid and the volume of urine excreted in the rat.

Table 3. *Effect of the intraperitoneal injection of sodium benzoate on the concentration of peptide non-protein glycine in tissues of the rat*

Each determination was carried out on pooled material from three rats. Tissues were examined 3 hr. after injection of 1 ml. of sodium benzoate (dose 340–400 μmoles/100 g. body wt.). Control rats were injected with water (1 ml.). P is the probability that there is no significant change in concentration after administration of benzoate.

	Control		After benzoate		
Tissue	No. of expts.	Mean ± S.D. (range) (μmoles of glycine/100 g.)	No. of expts.	Mean ± S.D. (range) (μmoles of glycine/100 g.)	P
Liver	3	778 ± 157 (600–906)	3	856 ± 200 (671–1071)	>0·1
Blood	2	91 ± 16 (82, 100)	2	88 ± 32 (66, 110)	>0·1
Kidney	3	357 ± 146 (193–478)	3	492 ± 93 (414–600)	>0·1
Muscle	3	514 ± 64 (464–578)	3	528 ± 14 (421–685)	>0·1
Intestine	3	404 ± 50 (364–457)	2	278 ± 7 (270, 286)	>0·05

DISCUSSION

The information summarized in Table 1 suggests that liver is the most important site of hippuric acid synthesis in the rat. The kidney of the rat can also effect synthesis of hippuric acid *in vitro* but there appears to be some disagreement as to the relative activities of the two organs in this respect (Borsook & Dubnoff, 1940; Cohen & McGilvery, 1946). The only tissue other than liver in which a significant decrease of free glycine was observed was blood. Since there is no evidence that hippuric acid can be synthesized by blood, it may be concluded that the decrease in blood free glycine is a consequence of either transport of glycine into the liver or retention of glycine by the liver.

From the steady levels of liver free glycine, before and after administration of benzoate, the turnover rate of liver free glycine can be calculated if it be assumed that (*a*) the rate at which free glycine is introduced into the liver is constant, (*b*) the rates of the reactions which remove glycine are proportional to the level of liver free glycine and (*c*) the liver is metabolically homogeneous (see Henriques, Henriques & Neuberger, 1955). The justification for (*a*) and (*b*) is examined later; (*c*), though probably incorrect, is commonly assumed in investigations similar to the present one.

If the first two assumptions are incorrect, the value of the turnover rate may, in theory, be either greater or less than the calculation indicates, but in practice errors are most likely to result in an underestimate of the turnover rate. The following values should therefore be regarded as minimal.

It is shown in the Appendix that on the above assumptions the turnover rate, **v**, is given by the equation

$$\mathbf{v} = v_h g_1/(g_1 - g_2),$$

where v_h is the rate of formation of hippuric acid, and g_1 and g_2 are the steady levels of liver free glycine before and after administration of benzoate. Substitution of the values of g_1 and g_2 recorded in Table 1, and the mean value found for v_h (36 μmoles/100 g. body wt./hr.), yields a value for **v** of 78 μmoles/100 g. body wt./hr. Since the livers used averaged 4 % of the weight of the rats, and their glycine level was 268 μmoles/100 g. of liver (Table 1) it follows that the level of liver free glycine (in the absence of benzoate) is 10·7 μmoles/100 g. body wt., so that the relative turnover is $78/10{\cdot}7 = 7{\cdot}3$ hr.$^{-1}$, i.e. the half-life period is approximately 6 min.

Assumptions made in calculating turnover rates

Input of glycine. The validity of the above calculations depends to some extent on the correctness of the assumptions made. There is evidence which suggests that administration of benzoate does not change the rate of introduction of free glycine into the liver. The results of Arnstein & Neuberger (1953) show that the gross rate of synthesis of glycine from serine and other sources did not vary with the dietary supply of glycine. There is evidence (unpublished results) that after administration of benzoate the liver-serine level remains constant. It is likely, therefore, that the gross rate at which serine is converted into glycine is also constant. (The net rate of conversion, which depends also on glycine concentration, is believed to vary.) The fact that the growth of young rats is arrested by the incorporation of benzoate into a low-glycine diet, and restored by the addition of glycine (Griffith, 1929, 1930; White, 1941) suggests that any increase in the rate of synthesis of glycine caused by a more specific effect of benzoate must be limited in extent, if it occurs at all. Other evidence that benzoate does not cause an appreciable increase in the production of glycine, by synthesis or by liberation from protein or other compounds, is provided by the work of Arnstein & Neuberger (1951), who found that administration of labelled glycine to the rat resulted in a rapid equilibration with the 'first glycine pool', after which the content of labelled glycine in the liver, as excreted in hippuric acid, decreased slowly. The rate of this decrease corresponded to the introduction of unlabelled glycine to the liver by synthesis and liberation at a rate of only 20 μmoles/100 g. body wt./hr. in the presence of benzoate. Finally, no evidence has been found that benzoate results in the depletion of glycine in peptides or liver proteins (cf. p. 578). Arnstein & Neuberger (1951) reported that they were unable to find a significant reduction in the total protein nitrogen of rat liver after the administration of benzoate.

It is shown in the present paper that after administration of benzoate, the level of blood free glycine decreases. If the rat resembles the rabbit in having no effective permeability barrier to the transport of free glycine between liver and blood, the transfer of free glycine to the liver will decrease by approximately 20 % (Table 1). Since exchange of free glycine between liver and blood accounts for about 20–30 % of the turnover of liver free glycine, neglect of the decrease in the level of blood free glycine would cause an error of approximately 5 %.

Output of glycine. It has been assumed that the processes by which free glycine is lost from liver vary in rate with the glycine level. This is known to be so for some reactions, e.g. serine formation by way of formaldehyde (Arnstein & Neuberger, 1953), benzoate conjugation (Bray *et al.* 1951), and also for transport processes, since the free-glycine content of liver would otherwise be unable to rise on administration of glycine or the blood free glycine to fall on administration of benzoate. The fact that on administration of benzoate a new steady level of

free glycine is attained, with a value about one-half that of the original level, shows that the extra loss of glycine due to hippuric acid formation is checked by the lowering of the glycine level, and hence that an appreciable proportion of the processes by which glycine is removed from the pool of free glycine in the liver vary in rate with glycine level. It is shown in the Appendix that, provided an appreciable fraction of the glycine is removed by processes with first-order kinetics, the errors in the deduction of turnover rate due to the operation of reactions of zero order with respect to glycine are not large and would result in the turnover rate being underestimated. It is unlikely that an appreciable proportion of the output is by way of processes which follow kinetics of orders greater than one with respect to glycine, and hence that the calculated turnover rate is too large.

The source of glycine for hippuric acid conjugation

It is now possible to consider the magnitude of the rates of some reactions supplying and removing glycine, and of the glycine stores available. It is known that the conjugating moiety in hippuric acid synthesis is free glycine, and that the benzoate is 'activated' before conjugation (cf. Schachter & Taggart, 1953). The liver free glycine is about 10·7 μmoles/100 g. body wt., while the rate of hippuric acid synthesis is 36 μmoles/100 g. It is evident that the pool of liver free glycine existing at the time of benzoate administration can supply glycine for only a small fraction of the usual doses (e.g. 50–600 μmoles/100 g. body wt.).

It is likely that plasma, and therefore liver, can withdraw free glycine from the remaining viscera quite readily. From the values for the concentration of tissue free glycine in Table 1 and those for other organs given by Krueger (1950), and from organ weights given by Donaldson (1924), it can be calculated that there are about 35 μmoles of free glycine/100 g. body wt. in the viscera and 86 μmoles of muscle. [On the basis of higher values for tissue free glycine Arnstein & Neuberger (1951) calculated 70–90 μmoles for viscera.] Muscle has relatively slight permeability (Henriques *et al.* 1955). Hence there is a store of approximately 35 μmoles of free glycine/100 g. body wt. available for conjugation, equivalent to only a small dose of benzoate. Arnstein & Neuberger (1951) calculated the size of a 'first glycine pool' in the rat to be 170 μmoles/100 g. body wt., but this is a maximum value, and the size is probably much less. (See Fig. 1, Arnstein & Neuberger, 1951.)

The turnover rate of rat-liver free glycine (78 μmoles/100 g. body wt./hr.) is made up of several components. The input due to transport from plasma can be calculated (assuming no permeability barrier, a liver 4 % of the body weight, a blood flow of 50–100 ml./100 g. of liver/hr., and a haematocrit value of 45 %) to be 13–26 μmoles/100 g. body wt./hr., corresponding to 17–33 % of the turnover rate. In the presence of benzoate, values of 10–20 μmoles are obtained. The contributions of peptide and proteins to the turnover rate are small. Thus by applying the 'replacement times' found for the rabbit (Henriques *et al.* 1955), it can be calculated that liver peptides (assumed to have the replacement time found for glutathione) contribute approx. 2 μmoles/100 g. body wt./hr., and liver proteins approx. 7 μmoles. Plasma proteins synthesized in the liver require about 4 μmoles/100 g. body wt./hr., but presumably do not return this directly.

The rate at which glycine is synthesized in the liver is not known, but since glycine is synthesized chiefly via serine (Arnstein & Keglević, 1956; Arnstein & Stanković, 1956) it presumably represents the difference between the rate of production of glycine from serine and of serine from glycine. The rapid equilibration of isotopically labelled glycine and serine suggests that these rates are high, and probably account for most of the remaining fraction of the liver turnover rate.

On the basis of the above considerations, the most probable sources of glycine for hippuric acid synthesis are as follows. In the first few minutes after administration of benzoate, part of the existing free glycine in the liver, and to a lesser extent in the blood, is consumed. Thereafter, the pool of liver free glycine is replenished from normal sources, i.e. by synthesis, by transport via blood, and by liberation of bound glycine. The level of liver free glycine falls until the rate of removal of glycine equals the rate of introduction and a new steady level is attained. The rate of removal of glycine by the normal processes of peptide and protein synthesis, degradation and transport is diminished. The relative extent to which the various removal processes are affected will depend on the reaction kinetics; thus the processes following first-order kinetics would be equally affected, whereas those following zero-order kinetics with respect to glycine would not be affected at all. No evidence has been found for the preferential depletion of the glycine of any constituent or of any tissue except liver and blood, so that it is likely that most of the removal processes are reduced to a comparable extent. The fall in blood free glycine which is due to a decline in the efflux of liver free glycine will lower the diffusion rate into all other organs, and, in effect, make the whole organism a source of glycine for conjugation. The relative contribution of these organs will depend on their permeability, mass and turnover rate.

Since only liver and blood are significantly depleted of free glycine, the replacement of any free

glycine lost from other tissues to liver must be readily accomplished, and the net rate of glycine transfer to liver must be a small fraction of the turnover rate of the free glycine of these tissues. The total rate of glycine synthesis, which Arnstein & Neuberger (1953) found to be 104 μmoles/100 g. body wt./hr. in immature rats and somewhat less (70 μmoles) in two adult rats, is evidently adequate for this purpose in the rats used in the present study. The inhibition by benzoate of the growth of young rats suggests, however, that there is only a small margin above sufficiency. This is borne out by a calculation made by Arnstein & Neuberger (1953), which shows that the requirement of glycine for synthetic purposes is almost as great as the rate of synthesis.

This discussion applies only to rats on a glycine-poor diet. When ample amounts of dietary glycine are available, hippuric acid synthesis will become simply one of the mechanisms for disposing of surplus glycine, comparable, for instance, to the degradation of glycine to formaldehyde (Arnstein & Neuberger, 1953).

APPENDIX

The relationship between the turnover rate for liver free glycine and the change in steady levels of free glycine can be deduced as follows.

Assuming that the input of free glycine to the liver, v_1, is constant, and the output is proportional to the glycine level, g, then

$$dg/dt = v_1 - kg, \qquad (1)$$

where k is a constant.

Let g_1 and k_1 be the values of g and k in the steady state in the absence of benzoate. Then

$$dg/dt = 0 \quad \text{and} \quad g_1 = v_1/k_1. \qquad (2)$$

After the administration of benzoate, the output of glycine is increased to $(k_1 + k_2)\, g$, where k_2 is the velocity constant for hippuric acid formation. The free glycine level falls to a new steady value, g_2, when

$$dg/dt = 0, \quad g_2 = v_1/(k_1 + k_2). \qquad (3)$$

From (2) and (3), the turnover rate $\mathbf{v}$ is given by

$$\mathbf{v} = v_1 = k_1 g_1 = (k_1 + k_2)\, g_2 = k_2 g_2 g_1/(g_1 - g_2) = v_h g_1/(g_1 - g_2), \qquad (4)$$

where v_h is the constant rate of formation of hippuric acid. The relative turnover is $\mathbf{v}/g$ (see Reiner, 1953) and the half-life period is $0{\cdot}7\, g/\mathbf{v}$.

If on administration of benzoate the input of free glycine is decreased, the turnover rate calculated from equation (4) will be too low. Thus if glycine input is decreased to αv_1, equation (3) becomes

$$g_2 = \alpha v_1/(k_1 + k_2),$$

and

$$\mathbf{v} = k_1 g_1 = v_h g_1/(\alpha g_1 - g_2).$$

Similarly, if the decline in output of free glycine is less than proportional to the glycine level, equation (4) gives too low a value for $\mathbf{v}$. Suppose that only a fraction, β, of the output is due to a set of reactions of first order with respect to glycine, and the remainder is due to zero-order reactions. Equation (1) then becomes

$$dg/dt = v_1 - kg - (1 - \beta)\, v_1 = \beta v_1 - kg,$$

and hence

$$\mathbf{v} = v_h g_1/\beta(g_1 - g_2).$$

The course of the transition between the two glycine levels may be derived as follows. After the administration of benzoate,

$$dg/dt = v_1 - (k_1 + k_2)\, g.$$

Integrating,

$$g = Ce^{-kt} + v_1/(k_1 + k_2).$$

The integration constant, C, may be evaluated, since when $t = 0$, $g = g_1 = v_1/k_1$. Hence

$$C = v_1/k_1 - v_1/(k_1 + k_2) = g_1 - g_2,$$

and

$$g - g_2 = (g_1 - g_2)\, e^{-(k_1 + k_2)t}.$$

A plot of $\log_{10}(g - g_2)$ and t should be linear, with a slope of $-(k_1 + k_2)/2{\cdot}3$ (see Fig. 3).

SUMMARY

1. The influence of the administration of benzoate upon the concentration of glycine in various tissues of the rat and the blood of the rabbit has been investigated. The administration of benzoate resulted in a fall in the concentration of free glycine in liver and blood but no significant change could be detected in kidney, intestine and skeletal muscle. No change in the concentration of liver-protein glycine could be observed.

2. After the administration of benzoate, the concentration of free glycine in liver and blood fell rapidly and reached an approximately steady low level which was maintained for as along as conjugation continued. The change in concentration was independent of the body level of benzoate until very low levels of benzoate were reached.

3. The significance of the results obtained is discussed with reference to the source of the glycine utilized for conjugation, and a value for the turnover rate of free glycine in the liver has been calculated. It is suggested that, in the rat under the experimental conditions described, a substantial proportion of the glycine used for conjugation is provided via the free glycine of the liver.

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The Isolation and Identification of Horse-Dandruff Allergen

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Although much work has been carried out on the characterization of the agents which elicit the symptoms of human hypersensitivity, a chemically pure allergen has not yet been isolated. This can be attributed to various difficulties encountered in fractionating the complex mixtures obtained by extraction of allergenic materials, as well as to the lack of accurate methods for assaying biological activity. Consequently, it is not known whether the activity of extracts is confined to a single constituent. Until this is established it is impossible to ascertain whether there is any underlying chemical structure peculiar to all types of allergen molecules.

Extracts of inhalant-type allergens, such as pollens, house dust and animal danders, have been found to contain multiple proteins, pigment and a wide range of substances of low molecular weight. From such starting materials, numerous attempts have been made to isolate pure constituents (see, for instance, the reviews of Augustin, 1952, 1955). In all cases, the active fractions (often several were isolated from one system) proved to be of protein nature, although most showed an unusual stability to heat treatment and some appeared even to resist proteolysis. The part played by prosthetic groups in the biological reactions of these materials is still uncertain. Most active fractions of plant origin have been found to contain 'bound' carbohydrate and, in some cases, pigment, but removal of these 'conjugated' substances often failed to reduce skin reactivity.

The work described here was undertaken with a view to identifying the allergen (or allergens) of horse dandruff and to obtaining a pure preparation which might prove useful in biochemical studies of the allergic reaction. Only the mildest of chemical procedures have been adopted in an attempt to preserve the native structure of the sensitizing substance. The drastic treatment applied to many allergenic extracts in the past has probably contributed to the difficulty of isolating pure constituents. In many cases the sole aim appears to have been the preparation of skin-reactive materials regardless of the deleterious action of the reagents employed (strong acid, strong alkali, organic solvents, etc.). Although such treatments sometimes apparently did not decrease the potency of the allergen, as indicated by prick testing of patients, they would tend to alter its chemical structure. This is particularly undesirable where the preparation of the allergen is required for immunochemical studies.

Squire (1950), using saline extracts of horse dandruff, achieved a partial purification of the protein constituents by fractionation with sodium sulphate. In addition, quantitative skin-testing procedures were defined. The solubility and serological characteristics of the active protein suggest that the dandruff allergen might be a modified form of horse-serum albumin. This possibility has been borne in mind throughout the present study and, where possible, the properties of the dandruff antigens have been compared with those of crystalline horse-serum albumin in the hope of providing a chemical explanation for this intra-species relationship.

MATERIALS AND METHODS

Preparation of horse-dandruff extract. A suspension of horse brushings in acetone at 1° was poured through a large Büchner funnel to separate the hair from the dandruff (mainly epidermal scales). After standing in cold acetone for 1 hr. to remove fats, the dandruff was separated by filtration with suction and dried over P_2O_5 in a vacuum desiccator. A soft grey powder was obtained. Portions (100 g.) were extracted by trituration with 0·85% (w/v) saline (1 l.), toluene (100 ml.) being added as preservative. The resultant thick cream was stored, with occasional stirring, at 1° for 3 days, after which insoluble material (mainly keratin) was removed by centrifuging (10 000 rev./min.) at 5°, the dark-brown supernatant being used for many tests and termed 'crude dandruff extract'.

As a preliminary purification process, substances of low molecular weight such as peptides, amino acids, urea, sugars and salts (comprising about 40% of the total solids) were removed from the crude extract by dialysing portions (100 ml.) in Vico cellophan sacs at 1° for 3 days against large volumes (5 l.) of tap water and then for 1 day against distilled water. 'Dialysed extracts' obtained in this way were light brown and opalescent, containing about 1% (w/v) of protein.

More protein (about 25% of that initially extracted) could be obtained by a second extraction of the defatted powder.

Determination of protein concentration. Total nitrogen was estimated by the micro-Kjeldahl technique of Ma & Zuazaga (1942), distillations being carried out in a Markham (1942) type of apparatus. Protein solutions were digested overnight.

Non-protein nitrogen was estimated after precipitation of the protein by the addition of an equal volume of 10% (w/v) trichloroacetic acid.

In calculating protein concentrations from the nitrogen contents of solutions, the conversion factor 6·25 was always used.

Total solids were estimated by heating duplicate portions of solution in a hot-air oven at 110° to constant weights.

Ultraviolet-absorption measurements. Cells 1 cm. in width were used, measurements being made in an SP. 500 spectrophotometer (Unicam Instruments Ltd.).

Electrophoretic analysis

Free-solution electrophoresis. Barbitone buffer, pH 8·6; *I*, 0·1 (sodium diethylbarbiturate, 0·1 M; diethylbarbituric acid, 0·2 M), was mainly used. Specific conductivities of diffusates were measured at 0·5°.

Analyses were carried out at 0·5° in a Tiselius apparatus, incorporating a Philpot–Svensson optical system. Monochromatic light ($\lambda = 546$ mμ.) was used, schlieren patterns being analysed by the method of dropping perpendiculars from minima separating the peaks (Tiselius & Kabat, 1939) and estimating the enclosed areas by the method of 'counting squares'.

In calculating the potential gradient within the cell the specific conductivity of the protein solution was assumed to be equal to that of the diffusate. The small correction theoretically required for volume change in the electrode vessels was neglected.

Horizontal paper-strip electrophoresis. The technique adopted was a development of that originated by Turba & Enenkel (1950) described fully by Hardwicke (1954). In most experiments, strips (39 cm. × 6–9 cm.) of Whatman no. 3 MM paper were used. Portions (0·01 ml.) of 1% (w/v) protein solutions were applied to the strip. Barbitone buffer (pH 8·6; *I*, 0·05) was most commonly used as solvent and a current of about 2 mA/strip was passed for about 16 hr. at room temperature. Protein was located by staining as described by Hardwicke.

Relative protein concentrations were determined by eluting the dye from segments of paper containing the separated components with 0·01 N-NaOH (10 ml.) for 30 min. (according to the method of Kunkel & Tiselius, 1951) and then measuring the optical densities of the eluates at 600 mμ. in a Hilger Spekker absorptiometer. The fading of the dye in this eluting fluid reported by Franglen & Martin (1954) was not encountered, provided that measurements were made within 1 hr. after elution.

Only narrow side strips (1 cm. wide) of paper, each carrying approx. 15% of the total protein, were stained in preparative runs to locate the proteins on the unstained centre section. Appropriately cut segments were eluted with 0·85% saline for 24 hr.

Preparative zone electrophoresis. The vertical starch-column method of Flodin & Porath (1954) was used to prepare sufficiently large quantities of electrophoretic components for chemical, immunological and biological analyses. Commercial potato starch, thoroughly washed with 0·85% saline and then with barbitone buffer (pH 8·6; *I*, 0·05) at 1° was used as the solid supporting medium. Samples containing about 300 mg. of protein in 6 ml. of buffer were separated under a potential of 600 v d.c. (giving a current of 20 mA) for about 16 hr.

The column was eluted with buffer and eluates (2·5 ml.) were collected by an automatic fraction collector (Towers and Co. Ltd.). Distribution of electrophoretic zones was revealed by measuring the absorptions ($\lambda = 277{\cdot}5$ mμ.) of portions (0·2 ml.) of the eluates diluted with water (3 ml.) against a solvent blank consisting of barbitone buffer similarly diluted.

Carbohydrate estimations. The Molisch reaction, with 1% (w/v) ethanolic α-naphthol solution and conc. H_2SO_4, was used as a rapid preliminary test for carbohydrate.

Carbohydrate bound to proteins separated electrophoretically on paper was revealed by staining with periodic acid–Schiff's reagent, according to the method of Köiw & Grönwall (1952). For recording the results it was necessary to photograph almost immediately the pink bands obtained, as background colour rapidly developed.

Quantitative measurements of carbohydrate. A modified orcinol colorimetric technique, applied by previous workers (Rimington & Van den Ende, 1940; Friedmann, 1949) to the estimation of hexose in horse-serum glycoproteins, was used to determine the hexose contents of horse-dandruff protein fractions. Absorption measurements were made at 520 mμ. in a Unicam spectrophotometer against the appropriate reagent blank. Equimolecular proportions of galactose and mannose were used for construction of a standard curve.

Phosphorus estimation. The Fiske & Subbarow (1925) colorimetric method was applied to the estimation of protein-bound phosphorus.

Chromatographic analysis. One-dimensional ascending-partition chromatography was employed, pyridine–amyl alcohol–water (35:35:30, by vol.) and pyridine–ethyl acetate–water (20:40:40, by vol.) proving to be satisfactory solvent systems.

Samples (10 mg.) of glycoprotein were hydrolysed by heating with conc. H_2SO_4 (1 ml.) in sealed tubes in a steam oven for 4 hr., according to the method of Werner & Odin (1949). Sulphate was removed from the hydrolysates by addition of $Ba(OH)_2$.

Chromatograms were run on Whatman no. 1 paper at 27° for 1–3 days and then sprayed with aniline hydrogen oxalate (Horrocks & Manning, 1949). A modified Morgan-Elson reagent (Partridge, 1948) was used to reveal hexosamines and *N*-acetyl hexosamines.

Molecular-weight determination. The apparatus used was similar to that employed by Adair (1949) in the determination of the osmotic pressures of haemoglobin solutions, involving measurement of the osmotic head in terms of toluene coloured with dye. Semi-permeable collodion membranes were prepared according to the method of Alexander & Johnson (1949). Osmotic pressure measurements were made at 1° on protein solutions of varying concentrations (up to 1%, w/v) in buffer solutions (*I*, 0·2) at the isoelectric pH of the protein. Latterly, however, osmotic pressure measurements were made in an electronic colloid osmometer (Rowe, 1953).

Immunological techniques. Antisera were prepared by injecting rabbits intravenously with alum-precipitated protein from concentrated [3% (w/v) protein] crude horse-dandruff extract (Kabat & Mayer, 1948). Five days after the last injection of the 3 weeks' course of immunization, the rabbits were bled from the ear.

'Ring' tests, performed in narrow glass tubes (2 mm. internal diam.), proved useful in selection of suitable antisera for subsequent gel-diffusion analyses.

The horizontal gel-diffusion technique, evolved by Ouchterlony (1948) and Elek (1949), was used for comparing the protein components in horse-dandruff fractions. The distances between the edges of the antigen and antiserum agar was usually 11 mm., and the plates were incubated at 37° for 1–2 days or, in some cases, kept at room temperature. To obtain a permanent record of the precipitin lines, the agar slabs were fixed in buffered (pH 7·4) formol saline and stained with chlorazol black E (Gell, 1955).

Further information was provided by a modification of the Grabar & Williams (1953) immuno-electrophoretic technique. Solutions (1%, w/v) of agar in barbitone buffer (pH 8·6; *I*, 0·05) were used to form the gels, through which a current of about 3 mA was passed for 10 hr. Antiserum was then placed in a trough cut at a suitable distance from, and parallel with, the line of electrophoretic separation.

Preparation of crystalline horse-serum albumin reference substance. A crude crystalline albumin fraction was prepared by the method of Adair & Robinson (1930). After three recrystallizations it was dialysed until salt-free, and freeze-dried. The product was found to contain 0·3% of hexose and showed a major and one minor precipitin line in an agar-diffusion test of a 1% (w/v) solution with rabbit antiserum raised against whole horse serum.

Assay of allergenic activity. The biological activities of centrifugally Seitz-filtered dandruff preparations were determined by quantitative prick testing of horse-sensitive human patients. Duplicate tests were made by pricking drops of protein solutions (in 0·85% saline), suitably diluted after estimation of their nitrogen contents, into selected sites on the patient's forearm. After an interval of 10 min., the outlines of the wheals were traced in ink on stiff celluloid. The area of the enlarged tracings were determined by the method of 'counting squares' and used for calculating accurate mean wheal diameters.

Squire (1950) demonstrated the reliability of this method by repeated testing with histamine and subjecting the results to an analysis of variance, which indicated a coefficient of variation of about 16% for a single measurement (provided that suitable precautions in the selection of body site and dose were observed). He showed also that over a wheal size range of 1–6 mm. the relation between wheal size and the logarithm of the histamine concentration is approximately linear.

Subjects. A group of six male subjects (aged 9–13) were selected, from patients suffering with bronchial asthma of sufficient severity to warrant regular attendance at a schools' asthma clinic, because of their marked skin reactivities to horse-dandruff extract (Bencard reagent). In addition, they gave positive skin tests to other common allergens such as pollens, dust, etc.

RESULTS

Preliminary studies on the chemical composition of horse-dandruff extract

Protein. Dialysed dandruff extract exhibited the opalescence of a typical protein solution and was found to have the usual type of protein ultraviolet-absorption spectrum (Fig. 1*A*). It was possible to

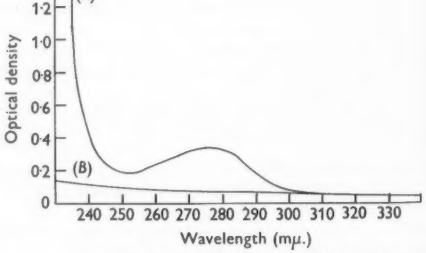

Fig. 1. Ultraviolet-absorption spectrum of dialysed horse-dandruff extract (approx. 50 mg. %, protein solution) before (*A*) and after (*B*) deproteinization with 10% (w/v) trichloroacetic acid.

remove all detectable absorbing material by precipitation with 10% (w/v) trichloroacetic acid, the supernatant showing no specific absorption (Fig. 1*B*).

Of the common precipitants, phosphotungstic acid was found to be the most effective in removing protein from dialysed horse-dandruff extract. Sulphosalicylic acid precipitated 95% and trichloroacetic acid 92% of the protein nitrogen precipitated by phosphotungstic acid. There was good agreement between the amount of protein in the phosphotungstic acid precipitate (a 16% nitrogen content being assumed), namely 1·28 g./100 ml. of extract, and the total solids content of dialysed dandruff extract (1·31 g./100 ml.) when allowance was made for an ash content of 2·3%. Hence it was inferred that most of the non-dialysable material in horse dandruff was of protein nature.

The work of Squire (1950), who showed that whereas no appreciable loss of allergenic activity was caused by prolonged dialysis of dandruff extract all activity was removed by precipitation with trichloroacetic acid and other precipitants, indicated that skin reactivity was associated with this protein material.

Carbohydrate. Dialysed dandruff extract gave a positive Molisch reaction, contrary to the findings of Squire (1950). The problem was to decide whether this carbohydrate was conjugated with protein, present as free polysaccharide or to be found in both forms.

Carbohydrate was also detected in extract deproteinized with trichloroacetic acid but was precipitated completely by phosphotungstic acid. Attempts were made to obtain some quantitative measure of this constituent by application of a method used by Winzler, Devor, Mehl & Smyth (1948) to estimate the 'mucoprotein' in human plasma. In Table 1, the 'mucoprotein' contents of crude and dialysed dandruff extracts are compared with their total hexose contents, the differences giving the amounts of carbohydrate associated with protein precipitated by perchloric acid (or presumably by trichloroacetic acid). Although the results obtained were only semi-quantitative, owing to the difficulty of separating mucoprotein from the less soluble protein, it was evident that the major portion of the carbohydrate (determined as hexose) in dandruff extract was associated with the perchloric acid-precipitated (or heat-coagulated) protein.

Table 1. *Carbohydrate content of horse-dandruff extract*

Dandruff extract (2 ml.) was deproteinized by the addition of 0·75 M perchloric acid (8 ml.). After 10 min. the precipitates were separated by filtration through Whatman no. 540 paper and mucoprotein was precipitated from portions (5 ml.) of the filtrate by the addition of 5% (w/v) phosphotungstic acid in 2 N-HCl (1 ml.) in centrifuge tubes. The precipitates were separated after 15 min. by centrifuging (15 000 rev./min.), washed and dissolved in 80% sat. Na_2CO_3 (5 ml.) for analysis of their hexose contents.

Extract	Total hexose (mg./100 ml.)	Mucoprotein hexose (mg./100 ml.)	Difference (mg./100 ml.)
Crude	88·0	7·6	80·4
Dialysed	71·0	7·1*	63·9

* A value of 5·2 mg./100 ml. was obtained for mucoprotein precipitated from extract which had been deproteinized by heat coagulation at pH 4·0

Practically none of the so-called 'mucoprotein', which was found to have a hexose/nitrogen ratio of 1·38 (in crude extract), appeared to be removed from crude extract by dialysis. The possibility of this constituent possessing appreciable allergenic activity appeared unlikely, however, on account of Squire's finding that all activity was removable from dandruff extract by trichloroacetic acid precipitation. Moreover, this was later confirmed by testing a sample obtained from a full-scale fractionation of dialysed extract.

Approximately 19% of the total carbohydrate of crude dandruff extract was removed by dialysis (probably in the form of free sugars).

Phosphorus. Quantitative colorimetric analyses on acid digests of 1% (w/v) dandruff protein solution (reconstituted freeze-dried dialysed extract) revealed a protein-bound phosphorus content of less than 2 mg./100 g. On the basis of a low phosphorus content Squire (1950) had ruled out the possibility of dandruff extract containing significant amounts of nucleoprotein. This was confirmed by the ultraviolet-absorption measurements, which failed to indicate any specific absorption at the 260 mμ. wavelength (Fig. 1, curve *A*).

Pigment. The properties exhibited by the pigment in crude dandruff extract suggested that it might be melanin (probably originating from the horse's melanophores and removed from the dandruff powder during saline extraction). Approximately 40% was removed by the dialysis process.

Electrophoresis

Early workers (Longcope, O'Brien & Perlzweig, 1926) on the horse-dandruff-extract system found that a major allergenic fraction showed maximum precipitation between pH 3·2 and 3·8. Later Squire (1950) demonstrated maximal precipitation of the active material at pH 4·0. Hence alkaline buffers, in which the proteins would be expected to be of sufficiently high charge, were used in preliminary electrophoretic analysis in a Tiselius apparatus.

As phosphate buffer (pH 7·7) has proved better than barbitone (pH 8·6) for the electrophoretic

separation of horse-serum proteins, it was anticipated this would also prove superior in the analysis of horse-dandruff extract. Results of comparable analyses in buffers of similar ionic strength (0·1) showed that the converse was true. The index of symmetry (i.e. the ratio of the height of the major descending component to that of the major ascending component) determined from the schlieren patterns obtained after 198 min. electrophoresis in barbitone buffer was 0·81 as compared with a value of 0·44 after only 48 min. separation in the phosphate buffer. Thus anomalous boundary effects appeared to be much more pronounced in the phosphate system, nor were these effects reduced appreciably by carrying out the analysis in phosphate buffer of twice the original ionic strength (i.e. I, 0·2).

These results were, somewhat surprisingly, analogous to those obtained by Longsworth (1942) on electrophoretic analysis of human serum, barbitone (pH 8·6) giving a better separation than phosphate buffer (pH 7·7) of equivalent ionic strength. Longsworth was of the opinion that the most suitable solvent for the analysis of an animal serum was species-dependent.

Electrophoretic analysis in barbitone buffer (pH 8·6; I, 0·1) resulted in the separation of three components, lettered arbitrarily as a, b and c in order of decreasing mobility in the schlieren patterns shown in Fig. 2*A*. It will be noticed that the pigment moved with the slowest (c) component.

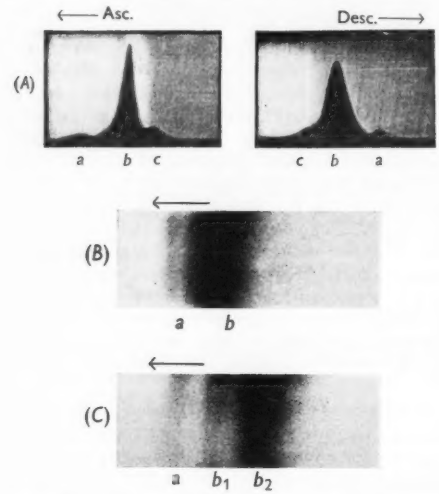

Fig. 2. Electrophoresis patterns of horse-dandruff extract [1 % (w/v) protein] in barbitone buffer (pH 8·6); (*A*) free solution analysis in buffer of ionic strength 0·1 (potential gradient = 7·31 v/cm., after 148 min.); (*B*) paper-strip analysis in buffer of ionic strength 0·05 (current = 2 mA for 16 hr.); (*C*) paper-strip analysis under conditions obtaining in previous experiment, illustrating the splitting of the major (b) component.

The relative concentrations of the components, which were all assumed to have the same refractive-index increment, were calculated from the ascending schlieren pattern as separation of the c component was far from complete in the descending limb. Paper-strip electrophoresis in barbitone buffer (pH 8·6; I, 0·05) also failed to separate this component (Fig. 2*B*). In some analyses, however, the major b component was split in the paper medium (Fig. 2*C*). Indeed, the slight asymmetry of the major peak noticed in free-solution patterns had suggested that the b component was heterogeneous. The faster of the two components was designated 'b_1' and the slower 'b_2'. The concentrations of the paper-strip components ($a = 5{\cdot}6\,\%$; $b = 85{\cdot}3\,\%$; $c = 9{\cdot}1\,\%$) showed good agreement with the corresponding values calculated from the ascending schlieren pattern ($a = 6{\cdot}0\,\%$; $b_1 = 18{\cdot}8\,\%$; $b_2 = 64{\cdot}4\,\%$; c, etc. $= 10{\cdot}8\,\%$) which suggest that the dandruff proteins did not differ greatly in their contents of non-protein prosthetic groups.

Determination of the isoelectric point of the major component

Mobilities of the b component were calculated from descending schlieren patterns obtained by electrophoresis of dandruff extract in three buffers of different pH. The mobility–pH curve intersected the zero-mobility line at pH 4·1, indicating an isoelectric value near to that obtained by the 'maximum-precipitation' method mentioned earlier. The horse-dandruff b component was found to have a mobility of $-8{\cdot}03 \times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$ in barbitone buffer (pH 8·6; I, 0·1); the corresponding value for horse-serum albumin (five times recrystallized) was $-6{\cdot}49$.

Solubility studies

Quantitative salt fractionations of horse-dandruff extract were undertaken with a view to determining the solubility limits of the constituent proteins, a necessary preliminary to their efficient separation.

Both phosphate buffer (pH 6·5) and saturated ammonium sulphate solution (adjusted to pH 7·0 with concentrated ammonia) were used as precipitants at controlled temperature. Use of phosphate enabled the protein contents of the supernatants to be determined by direct nitrogen estimations as well as affording a control on pH. The solubility curve obtained is shown in Fig. 3. Included, for comparison, in this figure is the curve obtained by plotting the ultraviolet absorptions ($\lambda = 277{\cdot}5$ mμ) of the supernatants against ionic strength of precipitants. Both curves revealed points of inflexion

at the same ionic strengths, indicating fractionation of three constituents.

Although the use of phosphate permitted the direct quantitative determination of precipitated protein by micro-Kjeldahl nitrogen analysis, ammonium sulphate has been more widely used as a protein precipitant in previous work and has become a standard against which to assess the solubilities of unknown proteins. A further quantitative fractionation therefore was carried out with ammonium sulphate, the amounts of proteins precipitated being determined by ultraviolet-absorption measurements on the supernatants. By allowing the protein–salt mixtures to come to equilibrium at 1° before filtration through Whatman no. 542 paper at room temperature, the possibility of further precipitation occurring in the spectrophotometer cell during measurement was avoided.

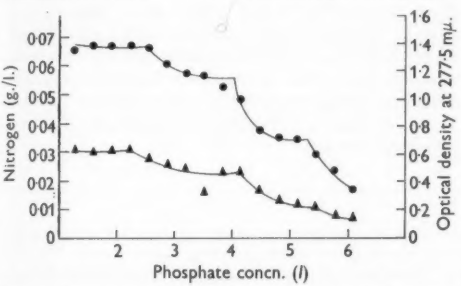

Fig. 3. Solubility curve of dialysed horse-dandruff extract in phosphate buffer, pH 6·5, at 25°. Varying amounts of 3M phosphate buffer (prepared from KH_2PO_4 and 4N-KOH) and 0·85% saline were added to portions (1 ml.) of dandruff extract to a final volume of 20 ml. in each case. After being heated in a water bath at 25±0·05° for 24 hr., and shaken occasionally, the precipitates were separated through Whatman no. 542 paper. Nitrogen contents (●) of the filtrate (diluted 10 times) were estimated by the micro-Kjeldahl technique, and optical densities (▲) measured in 1 cm. cells.

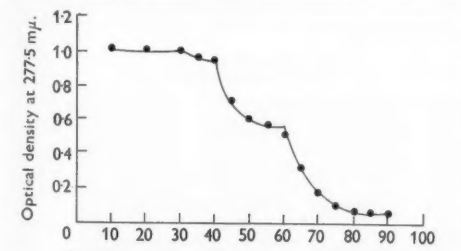

Fig. 4. Solubility curve of dialysed horse-dandruff extract in ammonium sulphate, pH 7·0, at 1°. Varying amounts of saturated ammonium sulphate solution (adjusted to pH 7·0 with conc. ammonia) and 0·85% saline were added to portions (1 ml.) of dandruff extract to a final volume of 20 ml. Mixtures were stored in a cold room for 24 hr., shaken occasionally, and precipitates separated by filtration (at 1°) through Whatman no. 542 paper. After the first portions of filtrates had been discarded, portions (1 ml.) were diluted (10 times) and optical densities measured in 1 cm. cells.

The solubility curve is shown in Fig. 4. Again, fractionation of three dandruff protein constituents is indicated. None of these showed a precipitation limit corresponding to 50% saturation with ammonium sulphate, whereas a similar experiment on horse serum indicated that the albumin was beginning to precipitate at this salt concentration.

Salt-fractionation of horse-dandruff extract

The conditions adopted in initial fractionations were similar to those commonly used in the salt-fractionation of horse serum. After separating the material precipitated by 50% saturated ammonium sulphate the soluble proteins were further fractionated by either lowering the pH or increasing the salt concentration. Precipitates and supernatants were analysed electrophoretically in free solution and, where possible, the relative concentrations of the separated components calculated from the enlarged schlieren patterns (Stanworth, 1953). A difficulty, particularly in cases of incomplete separation, was the identification of the various electrophoretic components in the different fractions.

As was to be expected, better fractionations were achieved by adopting the ammonium sulphate-concentration limits indicated by the quantitative fractionation with this salt (Fig. 4). A detailed scheme of a typical fractionation is shown in Fig. 5. The wet weights of the precipitates (after one washing with precipitant) are included in order to provide some indication of the distribution of protein amongst the various fractions. The largest portion of protein (about 50% of the total) was precipitated in fraction III between 55 and 80% saturation with ammonium sulphate.

The precipitates were taken up in tap water and dialysed at 1°, against repeated changes of tap water and finally against distilled water, until all the salt had been removed. Protein contents of the dialysed solutions were determined by micro-Kjeldahl nitrogen estimations and the concentrations adjusted to 1% (w/v), by dilution or evaporation in cellophan sacs in a stream of cool air, in preparation for subsequent analyses. All such solutions were stored at 1° for short periods and eventually freeze-dried.

Isolation of crystalline fraction

After numerous attempts, a specimen of crystalline dandruff protein was obtained, during the purification of crude fraction II (initially precipitated

between 42·5 and 55·0 % saturation with ammonium sulphate).

Twice-reprecipitated crude material (wet wt. 20 g.) was taken up in water (100 ml.), the solution being clarified by filtration through Whatman no. 542 paper. Saturated ammonium sulphate solution (72 ml.) was then added slowly to the solution at 1°, the sides of the beaker being scratched with a glass rod, until precipitation commenced. After standing for 3 hr. in the cold room the pigmented precipitate (wet wt. 10 g.) was separated by centrifuging. This was redissolved in water (100 ml.). Addition of saturated ammonium sulphate (76 ml.) at 1° brought down a slight precipitate, which was left standing overnight in the hope of adsorbing crystallization-inhibiting pigment. The precipitate was separated by filtration through Whatman no. 1 paper, leaving a pale-brown opalescent filtrate, on the surface of which there tended to form what appeared to be crystals. Storage of this solution (containing 43 % saturated ammonium sulphate) at 1° for another day resulted in the formation of a slight precipitate, which proved to be entirely crystalline on microscopic examination.

The crystals appeared to be of hexagonal form, thus differing from the trigonal horse-serum albumin crystals. They were shown to be anisotropic by examination under a polarizing microscope, and an aqueous solution gave a positive biuret reaction.

Further crystalline precipitates were obtained from the mother liquor. These were taken up in water, dialysed until salt-free and freeze-dried. More recently, a similar crystalline protein has been isolated from the ammonium sulphate solution washings (43 % saturated) of crude fraction I cooled to 1°, during a full-scale fractionation of horse-dandruff extract.

Saturated ammonium sulphate (adjusted to pH 7·0 with concentrated ammonia) was used as precipitant except in the precipitation of fraction IV, when solid salt was added. All precipitations were carried out in the cold room at 1°. The wet weights refer to the weights of the centrifuged precipitates after being washed once with approx. equal volumes of precipitant solutions of appropriate salt concentrations.

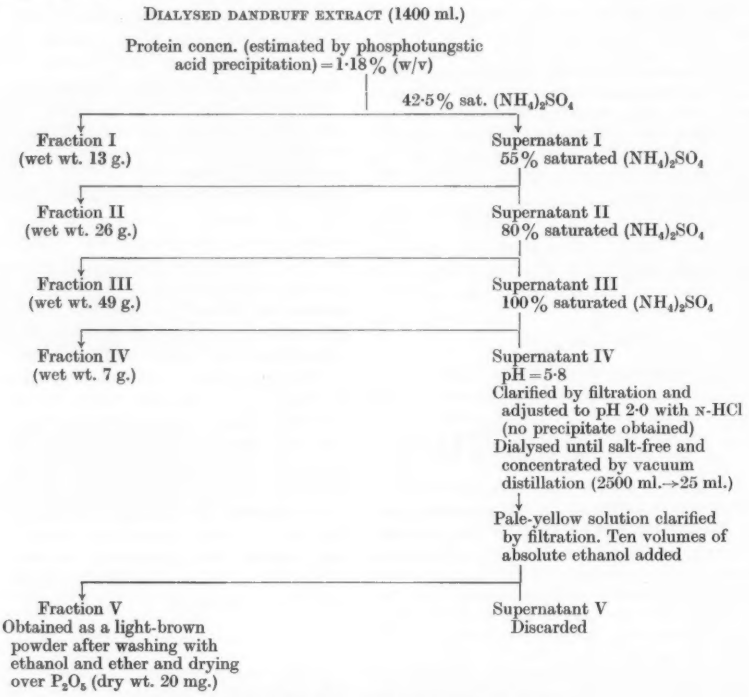
Dialysed dandruff extract (1400 ml.)
Protein concn. (estimated by phosphotungstic acid precipitation) = 1·18 % (w/v)
42·5 % sat. $(NH_4)_2SO_4$
Fraction I (wet wt. 13 g.)
Supernatant I 55 % saturated $(NH_4)_2SO_4$
Fraction II (wet wt. 26 g.)
Supernatant II 80 % saturated $(NH_4)_2SO_4$
Fraction III (wet wt. 49 g.)
Supernatant III 100 % saturated $(NH_4)_2SO_4$
Fraction IV (wet wt. 7 g.)
Supernatant IV pH = 5·8
Clarified by filtration and adjusted to pH 2·0 with N-HCl (no precipitate obtained)
Dialysed until salt-free and concentrated by vacuum distillation (2500 ml.→25 ml.)
Pale-yellow solution clarified by filtration. Ten volumes of absolute ethanol added
Fraction V
Obtained as a light-brown powder after washing with ethanol and ether and drying over P_2O_5 (dry wt. 20 mg.)
Supernatant V
Discarded

Fig. 5. Horse-dandruff extract fractionation scheme.

Electrophoretic analysis of fractions

The paper-strip technique was used in the electrophoretic analysis of the salt-precipitated protein fractions, enabling side-by-side comparison of mobilities to be made and thus overcoming the difficulty of identifying individual components, which was encountered in earlier free-solution analyses.

Results of a comparison of the electrophoretic patterns of total dialysed extract and its crude protein fractions (I–IV) on a wide sheet of Whatman no. 3 MM paper (22 cm. × 47 cm.) are illustrated diagrammatically in Fig. 6. (Paper-strip analysis of fraction V under similar conditions was not accomplished.) The system of nomenclature adopted in lettering the electrophoretic components of the other fractions was consistent with that applied to the components separated from whole extract (Fig. 2). The *c* and *e* components referred to in the diagram were only revealed by analysis of 6 % (w/v) solutions of certain fractions (notably fraction I), their mobilities relative to the other components being established by comparative electrophoresis on narrower (9 cm. wide) strips. It was evident that the two *b* components, which appeared to be closely related and often inseparable by electrophoresis of whole extract, had been separated by the ammonium sulphate fractionation. The difference between these constituents was further emphasized by staining paper-strip electrophoretic patterns of the various fractions with periodic acid–Schiff's reagent (Köiw & Grönwall, 1952), which showed the more soluble b_2 component to be rich in protein-bound carbohydrate (Fig. 7). On the other hand, no carbohydrate could be detected in the less-soluble b_1 component (predominant in fractions I and II). The faster *a* component contained a small amount of carbohydrate.

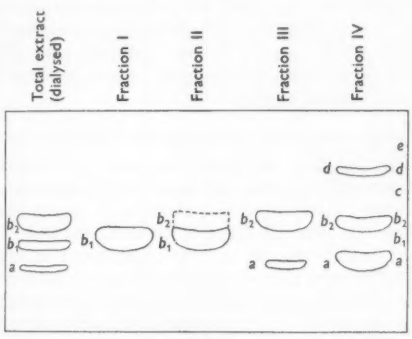

Fig. 6. Diagrammatic illustration of results obtained on comparison of the electrophoretic compositions of salt-precipitated dandruff protein fractions and total dialysed extract. Protein solutions (1 %, w/v) were analysed on a wide (22 cm. × 47 cm.) sheet of Whatman no. 3 MM paper in barbitone buffer (pH 8·6; *I*, 0·05; current 10 mA for 8 hr.).

The crystalline protein proved to be electrophoretically homogeneous as indicated by paper-strip analysis of a 3 % (w/v) solution. Its mobility on paper in barbitone buffer (pH = 8·6; *I*, 0·05) was identical with that of the b_2 component, although it differed from this protein in not containing any carbohydrate detectable by the periodic acid–Schiff's reagent.

Staining of paper-strip electrophoretic patterns with a saturated solution of oil red O in 60 % (w/v) ethanol (Durrum, Paul & Smith, 1952) failed to detect protein-bound lipid in any of the components.

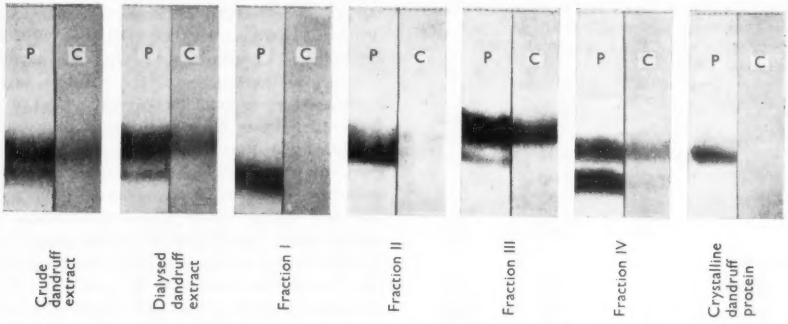

Fig. 7. Distribution of protein-bound carbohydrate in paper-electrophoresis patterns of horse-dandruff extract and protein fractions. (The *b* component is not split in the crude and dialysed extract patterns.) The dried strips were divided lengthwise, one section (P) being stained with bromophenol blue and the other (C) with periodic acid–Schiff's reagent.

Analysis of protein-bound carbohydrate

Paper-partition chromatographic analysis of the acid hydrolysates of crude glycoprotein fractions showed the protein-bound carbohydrate to be composed of galactose, mannose and glucosamine. In addition to these sugars, the highly soluble fraction V was found to contain a trace of glucose and another constituent, which was probably fucose.

It seemed significant that the sugar constituents of the horse-dandruff glycoprotein fractions were similar to those found in horse-serum proteins. Moreover, results obtained by application of a colorimetric method used by Friedmann (1949) in determining the relative concentrations of the hexose constituents of horse-serum proteins suggested that the galactose and mannose were present in similar proportions (namely 1:1) in the proteins from the two different sources. This involved measurement of the colour developed with orcinol reagent at two wavelengths, 470 and 520 mμ. The ratio of absorption at 470 mμ. to that at 520 mμ. then gave a measure of the galactose:mannose ratio in the mixture. Ratios obtained by measurements on solutions of most dandruff-protein fractions showed reasonable agreement with the mean ratio determined from similar measurements on varying dilutions of a standard solution containing equimolecular proportions of galactose and mannose (Table 2). Conclusive evidence from quantitative chromatographic analyses is, however, required.

Table 2. *Hexose contents of horse-dandruff proteins*

Hexose (galactose + mannose) contents of duplicate portions (1 ml.) of 0·25–1·00% (w/v) protein solutions were estimated by the orcinol technique, optical density measurements being made at 520 mμ. Measurements were also made at 470 mμ., the 470/520 mμ. absorption ratio providing an estimate of the relative concentrations of galactose and mannose comparable with the Ilford filter ratio 604/602 employed by Friedmann (1949).

Standard sugar solution (galactose + mannose, 1:1)	Total hexose content (γ/ml.)	Absorption ratio 470/520 mμ. (orcinol reagent)	Hexose/ nitrogen ratio
1	200	1·03	—
2	150	1·02	—
3	100	1·03	—
4	50	1·02	—
Protein fraction			
Dialysed extract	—	1·03	0·312
Fraction I	—	1·10	0·010
Fraction II	—	1·04	0·115
Fraction III	—	1·01	0·376
Fraction IV	—	1·00	0·245
Fraction V	—	—	8·620
Crystalline protein	—	1·10	0·013

The total hexose contents of the dandruff-protein fractions were also determined by the orcinol method, absorptions being measured at the 520 mμ. wavelength against the appropriate reagent blank. A calibration curve was constructed from the absorption values obtained by the measurements on dilutions of the standard galactose–mannose (1:1) solution at this wavelength. The carbohydrate contents expressed as hexose:nitrogen ratios in Table 3 were in the order expected from the results of the qualitative technique applied to the paper-electrophoretic patterns. According to the classification of Stacey (1946), the highly soluble fraction V which contained 48·0% of hexose and 5–6% of nitrogen would be termed a 'mucopolysaccharide'. Although it failed to react with biuret reagent, this could not be considered an indication of complete lack of peptide as polypeptide–polysaccharide complexes having low amino acid contents often show no reaction, particularly in the presence of contaminating pigment.

Assay of allergenic activities of dandruff fractions

The relative allergenic activity of dandruff extracts and fractions was assayed by prick testing patients with solutions containing a standard (0·1 g. %) concentration of protein. In Table 3, some results are set out for comparison of these activities with the composition of each specimen as determined by paper electrophoresis. It will be seen that the crude extract gave rise to wheals measuring from about 17 to 6 mm. in mean diameter, mainly according to the degree of sensitivity of the subjects tested. The results obtained with each of the other specimens have therefore been expressed as percentages of the mean diameter of the wheals elicited in duplicate tests with crude extract for each individual.

An analysis of variance in terms of patients, sites and fractions revealed that there was no significant variation ($P > 0{\cdot}20$) between the reactions of the different patients expressed in this way. This was interpreted as meaning (*a*) that the expression of results as percentages of the reaction size with a standard (crude extract) was valid, and (*b*) that the five individuals tested did not show significant variation in specific hypersensitivity to any particular dandruff-protein constituent. The contribution of the site to variance ($0{\cdot}10 > P > 0{\cdot}05$) did not quite reach the conventional level of significance. Nevertheless, as the sample analysed was small and as Squire (1950) has demonstrated a significant site variation by analysis of a larger number of prick tests with histamine on normal subjects, standard errors of the combined means were calculated by combining the variances due to sites 1 and 2. There was a marked variation in the mean reactivities of the various protein fractions.

Table 3. *Electrophoretic compositions and allergenic activities of salt-precipitated horse-dandruff protein fractions*

Relative concentrations of the electrophoretic constituents separated on filter paper were determined by quantitative elution of bromophenol blue in 0·01 N-NaOH (assuming all components bound dye to the same extent). Wheal diameters were calculated from tracings made 10 min. after pricking 0·1 % (w/v) Seitz-filtered protein solutions into duplicate sites on hypersensitive patients' right forearms. The results, expressed as percentages of the mean wheal diameters elicited by crude dandruff extract, are arranged in order of decreasing reactivity of the patients. There was a time interval of 14 days between the two series of tests performed on patient M.H. The standard errors of the combined means were obtained by combining the variances due to sites 1 and 2 (also in Tables 4 and 5).

Protein fraction	Relative concentrations of electrophoretic constituents (%)				Site	Wheal diameters elicited on duplicate sites								Standard error of mean
						Patient D.P. (mm.)		Patient M.H. (2nd test) (mm.)		Patient A.P. (mm.)		Patient M.H. (1st test) (mm.)		Patient R.V. (mm.)	Mean	
	a	b_1	b_2	Remainder (c, d, etc.)												
Crude extract	6·0	18·8	64·4	10·8	1	18·2	16·8	15·4	14·5	10·4	11·9	10·5	8·7	7·4	6·5	—
					2	15·3		13·5		13·4		6·9		5·6		
	% mean diameters elicited by crude extract															
Dialysed extract	4·9	25·3	56·7	13·1	1	121·4		89·7		108·4		103·4		84·6	90·8	14·0
					2	67·9		61·4		145·4		62·1		63·1		
Fraction I	0·0	74·6	0·0	25·4	1	27·4		37·2		26·9		0·0		47·7	36·6	8·5
					2	48·2		42·1		42·0		34·5		60·0		
Fraction II	0·0	71·8	13·4	14·8	1	32·7		34·5		47·9		0·0		81·5	45·3	16·5
					2	50·0		40·7		62·2		17·2		86·2		
Fraction III	6·8	8·7	78·3	6·2	1	52·4		89·7		84·0		118·4		95·6	87·6	14·5
					2	128·0		104·1		75·6		67·8		60·0		
Fraction IV	52·3	0·0	40·0	7·7	1	74·4		86·8		84·0		69·0		55·4	76·5	5·3
				(4·2 = d)	2	69·6		86·9		83·2		71·3		84·6		
Fraction V	—	—	—	—	1	0·0		—		0·0		—		—	—	—
					2	0·0		—		0·0		—		—	—	—

Comparison of the potencies of fractions I–IV (in terms of mean wheal diameter which is proportional to log dose of allergen) with their hexose/nitrogen ratios (Table 2) and electrophoretic compositions (Table 3) shows that the most potent fraction (III), possessing 96 % of the activity of the whole dialysed extract, contained the most hexose and a preponderance of the carbohydrate-rich b_2 constituent. (The mucopolysaccharide fraction V gave negative results in the two patients tested.) Summarizing, the fractions (III and IV) containing a high proportion of b_2 component were the most potent, whereas those (I and II) deficient in this protein were comparatively unreactive. (It should be mentioned that the value, 13·4 %, given for the relative concentration of b_2 component in fraction II can only be considered very approximate owing to the failure to effect a 'clean' electrophoretic separation of this component from the b_1 constituent predominant in this fraction.) There appears to be

Table 4. *Allergenic activities of partially purified horse-dandruff protein constituents*

Dialysed eluates containing each of the three main constituents of salt-precipitated (80–100 % saturated ammonium sulphate) fraction IV, separated by electrophoresis both on paper and in a starch column, were used in the prick testing of horse-sensitive patients.

A. Paper electrophoretic components. The low protein content of most eluates, even after concentration by evaporation in cellophan sacs in a current of air, prevented the use of the usual 0·1 % solutions in the biological testing.

Predominant electrophoretic component	Protein concn. of soln. tested (mg./100 ml.)	Site	Wheal diameters elicited in duplicate sites: Patient A.P. (mm.)	Patient D.P. (mm.)	Patient M.H. (mm.)	Combined mean (mm.)
Total fraction IV	100	1	12·4	13·3	8·7	12·6
		2	15·2	17·2	8·7	
a	20	1	4·5	4·0	1·6	3·5
		2	3·8	3·5	3·6	
b_2	22	1	8·4	8·3	6·4	7·8
		2	9·2	9·9	4·2	
d	118	1	1·8	0·0	4·0	1·7
		2	1·2	0·0	3·3	
'Tail'	20	1	1·3	2·5	0·0	0·7
		2	0·0	0·0	0·0	
Blank paper eluate	—	1	0·0	3·6	0·0	0·6
		2	0·0	2·8	0·0	

B. Starch electrophoretic components. The results of skin testing dialysed eluates from a starch column, diluted to contain 0·1 % of protein, are expressed as percentages of the mean wheal diameters elicited by the parent fraction (IV). A solution of the crystalline dandruff protein, of similar concentration, was tested at the same time.

Eluate no.	Predominant electrophoretic component	Site	Wheal diameters elicited on duplicate sites: Patient K.B.		Patient B.F.		Patient D.P.		Patient A.P.		Mean	Standard error of mean
Total fraction IV	$a+b_2+d$	1	4·2	9·3	10·3	13·3	15·3	17·9	6·5	4·4	—	—
		2	14·3		16·2		20·6		2·2			
			% mean diameters elicited by total fraction									
5–7	*a*	1	25·8		53·9		14·0		0·0		24·3	17·7
		2	23·7		75·9		0·0		0·0			
8–11	*a*	1	36·6		65·4		25·1		61·4		42·7	8·9
		2	37·6		53·4		23·5		38·6			
15	b_2	1	186·0		90·2		115·1		197·7		123·3	30·8
		2	143·0		112·8		79·9		161·4			
16–19	b_2	1	146·2		122·6		70·9		238·6		128·4	30·9
		2	110·8		106·0		83·8		152·3			
30	*d*	1	—		—		—		0·0		—	—
		2	—		—		—		0·0			
Crystalline protein	Same paper-electrophoretic mobility as b_2 component	1	36·6		79·7		53·1		36·4		47·2	10·2
		2	32·3		61·9		36·9		38·6			

no similar relationship between allergenic activity and the relative content of any of the other (*a*, *b*, *c*, *d*, etc.) electrophoretic components. For instance (cf. Table 3), if the *a* component were mainly responsible for allergenic activity, fraction IV (containing 52 % of this substance) would have been considerably more potent than any of the other fractions. Likewise this fraction would have shown the highest skin reactivity if the *d* component were allergenic. By similar reasoning, the preponderance of the b_1 component in the relatively unreactive fraction I rules out this substance as an important allergen. It seemed likely therefore that the b_2 electrophoretic component, comprising 64 % of the total protein in crude dandruff extract, was the main allergen. Moreover, the lack of a significant variation between the reactions of the individuals tested, as already mentioned, indicated that there was no evidence of specific hypersensitivity to any other proteins.

More clear-cut evidence in support of this finding was provided by skin testing more purified protein preparations. The results of tests on the electrophoretic components of fraction IV, separated either on filter paper or in a vertical starch column, are given in Table 4*A* and *B*, together with the allergenic activity of the crystalline dandruff protein which was tested at the same time. It was again found in both series of tests that the major part of the biological activity was associated with the b_2 constituent. However, the crystalline protein which on electrophoresis showed the same mobility as the b_2 component but which was deficient in carbohydrate was relatively unreactive on skin testing. The small amount of activity associated with the other components was probably due to contamination with b_2 component, undetected by paper-strip electrophoretic analysis. [Similar contamination might also account for the slight activity of fraction I (Table 3), which contained 75 % of the apparently unreactive b_1 electrophoretic component.]

It was interesting to find that the electrophoretic processes and subsequent elution from the supporting media did not reduce allergenic activity, nor did freeze-drying of the active protein prove deleterious.

Immunological analyses

Agar-diffusion techniques, which allow the side-by-side comparison of antigenic composition, provide a more sensitive test of protein purity than the physicochemical techniques already described. By applying these methods, horse-dandruff protein fractions which appeared homogeneous by paper electrophoresis were found to contain more than one antigen. In this way, trace contamination by the b_2 component was detected in the fractions of low but measurable allergenic activity.

A typical test is illustrated in Fig. 8, in which the antigenic compositions of the salt-precipitated dandruff-protein fractions are each compared with those of total extract. Several antigens could be detected in whole extracts tested with homologous antiserum. These were identified, as far as possible, by comparison with relatively pure protein preparations (obtained by preparative electrophoresis, etc.). A faint b_2 component line was found in the fraction I antigen pattern, which explained why this preparation possessed some allergenic activity although no b_2 component could be detected in this fraction by filter-paper electrophoresis. (The small amount of skin reactivity shown by the crystalline material could also be attributed to contamination with b_2 component similarly detected.)

An accurate assessment of antigen concentration cannot be made by this technique. Nevertheless, some measure can be obtained as both the distance of the precipitin line from the centre cup and its density are influenced by antigen concentration. From such considerations, no precipitin line other than that associated with the b_2 electrophoretic component could be assigned to the substance responsible for the allergenic activity of the various fractions. Furthermore, the b_2 line remained relatively unaffected when a solution of the potent allergenic fraction III was allowed to react with antiserum which had been first absorbed with weakly skin-reactive fraction I, a treatment which eliminated most of the other precipitin lines.

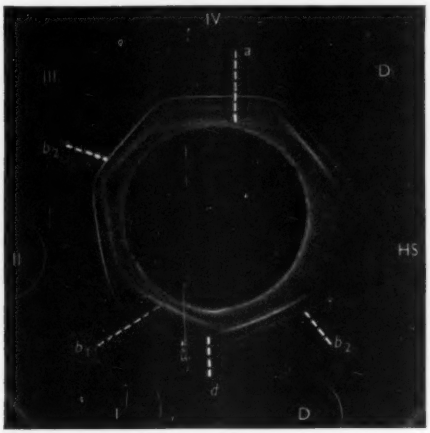

Fig. 8. Agar-diffusion precipitin patterns of dialysed dandruff extract (D) and salt-precipitated protein fractions (I–IV). Protein solutions (0·3 %) were tested with rabbit antiserum raised against crude dandruff extract. A 10 % (w/v) solution of total horse-serum proteins (HS) was also tested.

Purification of allergen

The gel-diffusion precipitin technique provided a sensitive guide to protein purity in subsequent attempts to prepare very pure allergen from the most active salt-precipitated fraction III. Sub-fractionation (both by salting-out and 're-solution' procedures) with ammonium sulphate achieved some degree of purification, as assessed by paper electrophoresis, although precipitin tests still revealed antigenic heterogeneity. Of six fractions assayed, that precipitated between 70 and 80% saturation with ammonium sulphate was found to be the most pure and proved to be a little more skin-reactive than crude horse-dandruff extract (possessing 115% of the activity of the whole). There was fairly close correlation between the activities of these fractions and their carbohydrate contents (expressed as hexose/nitrogen ratios), in keeping with earlier findings on the cruder salt-precipitated protein fractions.

Purer and more potent preparations were obtained, however, by vertical electrophoresis in starch columns. As much as 300 mg. of freeze-dried material was fractionated at a time by this method. After electrophoresis, the protein contents of the eluates (2·5 ml.) were determined by ultraviolet-absorption measurements against a buffer-solvent blank. The electrophoretic pattern obtained by analysis of salt-precipitated fraction III in barbitone buffer (pH 8·6) is shown in Fig. 9. It will be noticed that the protein contents of selected eluates, determined from micro-Kjeldahl nitrogen estimations on dialysed solutions, followed the optical-density curve closely.

Paper-electrophoretic analysis served as a useful check on the composition of eluates, before immunological analysis and assessment of their skin reactivities. The results of precipitin tests are shown in Fig. 10 (*A*). The most pure eluate (no. 11) of those tested was further analysed by the immuno-electrophoretic technique, Fig. 10 (*B*), and found to be considerably more pure than the parent fraction,

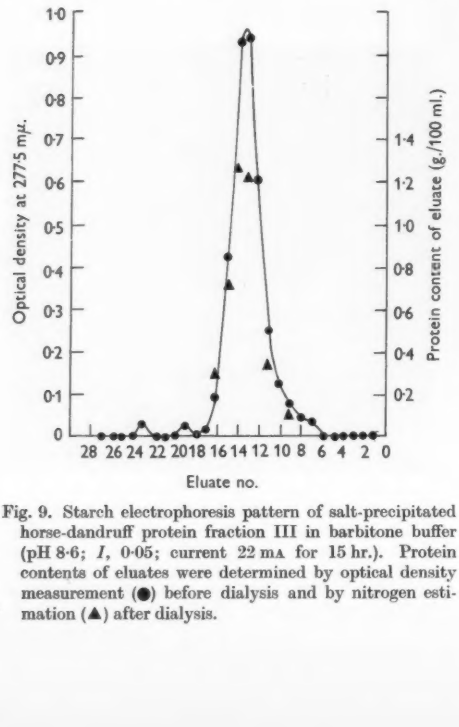

Fig. 9. Starch electrophoresis pattern of salt-precipitated horse-dandruff protein fraction III in barbitone buffer (pH 8·6; *I*, 0·05; current 22 mA for 15 hr.). Protein contents of eluates were determined by optical density measurement (●) before dialysis and by nitrogen estimation (▲) after dialysis.

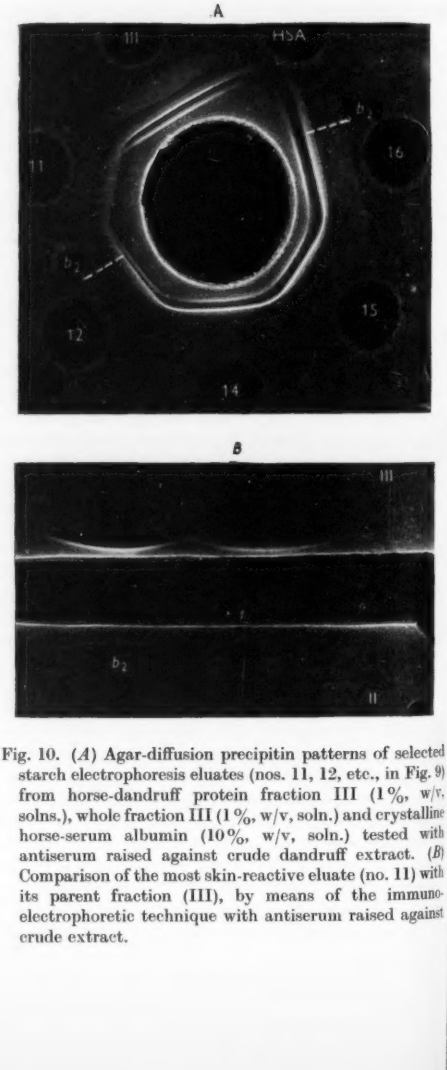

Fig. 10. (*A*) Agar-diffusion precipitin patterns of selected starch electrophoresis eluates (nos. 11, 12, etc., in Fig. 9) from horse-dandruff protein fraction III (1%, w/v, solns.), whole fraction III (1%, w/v, soln.) and crystalline horse-serum albumin (10%, w/v, soln.) tested with antiserum raised against crude dandruff extract. (*B*) Comparison of the most skin-reactive eluate (no. 11) with its parent fraction (III), by means of the immuno-electrophoretic technique with antiserum raised against crude extract.

Table 5. *Allergenic activities of starch-electrophoresis eluates of salt-precipitated (55–80 % saturated ammonium sulphate) fraction III*

Dialysed solutions containing 0·1 % protein were prick tested on horse-sensitive patients, the results being expressed as percentages of the mean wheal diameters elicited by the parent fraction.

Eluate no.	Predominant electrophoretic component	Site	Wheal diameters elicited on duplicate sites: Patient D.P. (mm.)	Patient K.B. (mm.)	Patient B.F. (mm.)	Mean	Standard error of mean
Total fraction III	$a+b_2$	1	12·6 } 14·6	5·3 } 7·9	5·9 } 6·8	—	—
		2	16·5 }	10·4 }	7·7 }		
			% mean diameters elicited by total fraction				
9	a	1	94·5	15·2	100·0 }	79·9	38·1
		2	57·5	13·2	158·8 }		
11	b_2	1	141·1	121·5	170·6 }	150·3	37·1
		2	78·8	160·8	219·1 }		
13	b_2	1	139·7	165·8	91·2 }	132·5	50·4
		2	60·3	100·0	238·2 }		
14	b_2	1	106·8	98·7	132·4 }	113·8	22·7
		2	93·2	119·0	(132·4) }		
15	b_2	1	89·7	169·6	148·5 }	120·8	27·1
		2	66·4	116·5	133·8 }		
16	b_2	1	80·1	100·0	216·2 }	117·5	12·0
		2	97·3	93·7	117·6 }		

a slower contaminating component (probably *c*) having been removed by the electrophoretic process, though appearing in increasing amounts in eluates 12–16. This eluate was also found to be the most potent, possessing 150 % of the activity of parent fraction III, although all the eluates corresponding to the main b_2 peak showed high allergenic activity (Table 5).

Preliminary chemical characterization of horse-dandruff allergen

Initial osmotic pressure (O.P.) measurements were made on a partially purified allergen preparation (contaminated with a little *a* component) at its isoelectric point in acetate buffer (*I*, 0·2) performed by means of a modified Adair technique. A mean mol.wt. value of about 42 000 (it was assumed that the protein contained 16 % of nitrogen, as micro-Kjeldahl estimations on solutions prepared from dried solid had suggested) was obtained from the results of measurements at varying protein concentrations (below 1 %, w/v) and extrapolating the osmotic pressure/concn.–concn. curve to zero concentration. In comparison a crystalline horse-serum albumin reference substance was found to have a mol.wt. of 71 000.

The relatively pure preparation obtained by starch electrophoresis, however, was found to have a mol.wt. of 34 000 in phosphate buffer (pH 7·4; *I*, 0·3) as compared with a value of 22 000 found for the crystalline dandruff protein. Each of these results was obtained by osmotic pressure measurements made in an electronic colloid osmometer (Rowe, 1953) on two solutions of different protein content (below 1 %, w/v).

Quantitative carbohydrate analysis, by the orcinol technique, showed the purified allergen preparation to contain 9·2 % of hexose (expressed as a percentage of protein) in the form of galactose and mannose. Its amino acid composition, revealed by preliminary qualitative chromatographic analysis, appeared similar to that of crystalline horse-serum albumin. Details of these and other analyses will be included in a full account of the allergen's chemical properties, to be reported later.

Serological relationship between horse dandruff and horse-serum antigens

The results obtained by application of the gel-diffusion precipitin technique suggested that the serological cross reaction between horse-dandruff protein and horse-serum albumin demonstrated by Squire (1950), who used the more laborious method of optimum proportions, might be due to traces of serum albumin and globulin admixed with the dandruff constituents.

Dandruff and serum antigens were tested with antisera raised against crude dandruff extract, crystalline horse-serum albumin and horse serum. The precipitin line produced by reaction of allergen with antiserum to crude dandruff extract was intersected by the horse-serum albumin precipitin line, which showed that there was no relationship between these two proteins. The serum-albumin

line, however, deviated and continued faintly close to the cup containing dialysed whole dandruff extract, which probably therefore also includes small amounts of this protein (a suggestion which explains why the antiserum to dandruff extract contains antibodies against horse-serum albumin). Conversely, lines due to reactions of concentrated dandruff extract [20 % (w/v) of protein] with antiserum to serum albumin were continuous with the homologous-antigen line. By similarly testing dandruff extract (and fractions derived from it) with antiserum raised against whole horse serum, lines continuous with the serum-albumin and with one of the serum-globulin lines were evident in the dandruff antigenic patterns; the latter line was well marked in the pattern of dandruff fraction I, but absent from fraction III which would seem to include traces of serum albumin.

Until these findings have been substantiated by tests with absorbed antisera, the presence of traces of serum protein in horse dandruff is tentatively concluded.

DISCUSSION

The results presented provide evidence that allergenic activity is confined to only one of the several protein constituents detected in horse-dandruff extract, namely the carbohydrate-rich b_2 electrophoretic component. Where paper electrophoresis fails to reveal this substance in preparations showing low but measurable activity, such as fractions rich in the a and b_1 components, traces of b_2 are detectable by the more sensitive immunological techniques.

There is no reason to suppose that other constituents are important allergens. Indeed, statistical analysis of the skin-test results indicates that there is no significant difference between the reactions of the limited number of subjects tested. Small differences apparent in some subjects' specificities (Tables 4 and 5) can be attributed to the inaccuracy of this method employed for assaying allergenic activity *in vivo*. In this respect, however, it should be noted that the coefficient of variation of the size of the wheals elicited by the b_2 component in the various subjects was of the order of 23 %, a value similar to the 16 % variation shown by Squire (1950) to be expected for a single measurement of the wheal elicited by pricking normal subjects with histamine. This rather wide spread in the size of wheals produced by a particular test solution has been responsible for the reluctance of many workers to express results in quantitative terms. Nevertheless, the use of the skin test as a quantitative assay of allergenic activity is considered to be valuable provided that the standard error of the results obtained with each test solution is given.

The relatively small increase (about 30 %) in skin reactivity achieved by extensive purification of the dandruff allergen explains why crude salt-precipitated protein fractions often proved to be little more reactive than the crude extract from which they were derived. By analogy, it might be possible to account for the failure of investigators of other allergen systems to effect any marked increases in activity by application of similar procedures. It appears that about 36 % of inactive protein (in addition to contaminating pigment) was removed from crude horse-dandruff extract by salt-precipitation and subsequent starch electrophoresis, resulting in the preparation of a white freeze-dried solid. An initial purification was, of course, effected by the prolonged dialysis of the crude extract, which removed about 40 % of the pigment in addition to substances of low molecular weights. The 10 % decrease in allergenic activity accompanying the dialysis process (cf. Table 3) is within the limits of error of the assay.

Ammonium sulphate fractionation of dandruff extract separated the allergenic b_2 component from the b_1 component to which it appeared to be 'combined' in solution. It is possible that the purification procedures adopted failed to free the allergen from other inactive substances. Is, for instance, the carbohydrate (9 % hexose) an essential part of the allergen molecule, or is it contained in a polysaccharide-rich contaminant? A small amount of unreactive mucopolysaccharide (isolated as fraction V from the supernatant obtained after saturation with ammonium sulphate) is present in horse-dandruff extract. It is unlikely, however, that any significant amount of this substance is contaminating the purified allergen preparation. Estimation of the mucopolysaccharide contents of crude dandruff protein fractions (by the method used by Winzler *et al.* in 1948 to estimate serum mucopolysaccharide) indicated that the major portion of the hexose found in such fractions was bound to perchloric acid- (or trichloroacetic acid-) precipitated protein. There was no correlation of skin reactivity with mucopolysaccharide content (Stanworth, 1953). Moreover, an attempt to combine the mucopolysaccharide constituent with the carbohydrate-deficient b_1 component (by adding fraction V to a solution of fraction I) proved unsuccessful, as indicated by paper-electrophoresis, nor did the mixture show any appreciable increase in skin reactivity.

Analysis of the carbohydrate contents of certain starch-electrophoresis eluates from salt-precipitated fractions indicated that the purer and more potent (on a nitrogen basis) the fraction, the higher must be its hexose/nitrogen ratio, which suggested that any contaminant of the allergen is carbohydrate-free protein. Such an assumption is substantiated by

the failure of carbohydrate-deficient crystalline protein, of similar electrophoretic mobility to the b_2 component, to show any appreciable skin reactivity. Furthermore, there is no evidence from precipitin tests that the crystalline protein (molecular weight 22000) is allergen deprived of carbohydrate.

Hence it is considered that horse-dandruff allergen contains a carbohydrate prosthetic group, in common with many other inhalant-type allergens. On account of its predominant protein-like properties, it would be termed a 'mucoprotein' according to the classification of glycoproteins proposed by Stacey (1946).

It would be tempting to explain the supposed serological cross-reactivity between horse-dandruff allergen and horse-serum albumin (Squire, 1950) in terms of common sugar constituents (galactose, mannose and glucosamine). According to agar-diffusion precipitin tests, however, the two proteins are unrelated. On the other hand, a contaminating antigen in the crystalline horse-serum albumin preparation (containing 0·3 % of hexose) appears to cross-react with the allergen (illustrated in Fig. 10*A*). Possibly this reaction is due to a serological relationship between the allergen and one of the horse-serum glycoprotein constituents such as seromucoid, which has been shown to contain 10·7 % of carbohydrate in the form of *N*-acetylglucosamine, D-galactose and D-mannose (Rimington & Van den Ende, 1940). These highly soluble substances tend to be co-precipitated with the albumin during salt-fractionation of serum.

Nevertheless, the apparent admixture of traces of horse-serum albumin and globulin with the dandruff constituents offers an alternative and simpler explanation of the serological relationship between the dandruff and serum antigens. It will be necessary to confirm these preliminary findings by carrying out more extensive precipitin tests with absorbed antisera. Squire (1950) rejected this mechanism on the basis of results obtained from skin tests on horse-sensitive asthmatics, and from the precipitin method of optimum proportions which does not allow the identification of antigens by direct comparison of their precipitin lines.

Although the isolation of an antigenically pure skin-reactive substance from horse dandruff is not claimed (extensive agar-diffusion tests on the most pure preparation revealed faint precipitin lines due to trace contaminants) the allergen preparation obtained is considered to be more pure than any other inhalant-type allergen yet isolated. Furthermore, all tests carried out were consistent with the hypothesis that skin reactivity is confined to this one constituent. In contrast, investigators of other allergen systems have found that activity is associated with several constituents. Augustin (1953), for instance, has fractionated an ultrafiltrate of crude grass-pollen extract into four skin-reactive protein complexes, differing in electrophoretic mobility and carbohydrate and pigment content but apparently possessing the same qualitative composition. Multiple active peptide–carbohydrate complexes were fractionated chromatographically from dialysed ragweed-pollen extract by Frankel, Johnson, Schiele, Bukantz & Alexander (1955). House-dust extract has also been fractionated, by electrophoresis, into a pigmented and non-pigmented component of similar allergenic activity, both of which were revealed in a 'standard purified allergen' obtained by acid or acetone precipitation (Rimington, Stillwell & Maunsell, 1947). Recently Silver & Bookman (1956) have described, in a preliminary report, the paper-electrophoretic fractionation of acetone-precipitated horse-dandruff material into two major components, the slower of which was associated with skin reactivity. These workers estimated the molecular weight of the dandruff antigen to be approximately 40000 from sedimentation measurements. In none of these cases, however, has the preparation of a chemically pure allergen been satisfactorily demonstrated.

A series of skin tests with the purified freeze-dried horse-dandruff allergen preparation is being planned in order to assess its suitability as a solid standard for materials used in the skin testing of horse-sensitive asthmatics. It is intended, also, to use this protein for hyposensitization purposes. Until larger numbers of patients have been tested quantitatively, however, it may be wise to carry out skin tests both with crude and with purified allergens in case some individuals are sensitive to some minor component, as is evidently the case with the horse serum-albumin component.

SUMMARY

1. The composition of horse-dandruff extract has been studied by means of physicochemical and immunochemical techniques, which have revealed the presence of at least seven protein or polypeptide constituents containing varying proportions of conjugated carbohydrate in addition to pigment and dialysable substances.

2. Electrophoretic analyses, both in solution and on paper, provided measures of the relative concentrations of the major protein constituents, which were lettered arbitrarily in order of decreasing mobility in veronal buffer at pH 8·6. Staining of the paper-strip patterns with periodic acid–Schiff's reagent revealed the distribution of protein-bound carbohydrate.

3. Fractional precipitation with saturated ammonium sulphate solution, between limits indicated

by preliminary quantitative fractionations, provided a series of precipitates containing varying proportions of the electrophoretic components.

4. All fractions were assayed by prick testing horse-sensitive asthmatics and allergenic activity was found to be associated with a single carbohydrate-rich constituent (designated 'b_2'), predominant in the fraction precipitated between 55 and 80% saturation with ammonium sulphate and comprising 64% of the total protein in whole dandruff extract. Additional evidence as to the nature of the allergen was obtained from skin tests with relatively pure constituents isolated by preparative electrophoresis on paper and in a starch column.

5. The precipitin line due to the allergen was identified in antigen patterns obtained by the horizontal gel-diffusion technique and detected in preparations of low skin reactivity, which appeared to be allergen-free according to the less sensitive electrophoretic analysis.

6. A very soluble mucopolysaccharide fraction (containing 48% of hexose), and a crystalline protein (of similar electrophoretic mobility on paper to the allergen, but containing only 0·2% of hexose), were found to be inactive.

7. A highly purified allergen preparation was obtained by the starch electrophoresis of the allergen-rich salt-precipitated fraction.

8. Preliminary chemical analyses showed the allergen to contain 9% of hexose in the form of galactose and mannose, in addition to all the common amino acids. Its molecular weight, determined from osmotic pressure measurements, was found to be 34 000.

9. Evidence is provided that the serological relationship between horse dandruff and horse-serum antigens is probably due to the admixture of serum albumin and globulin with the dandruff constituents.

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β-Glucuronidase Activity in Lymphatic Tissues of the Rat after X-Ray Irradiation of the Whole Body

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As communicated in a preceding paper (Pellegrino & Villani, 1956) increase in β-glucuronidase activity has been observed in lymphatic tissue during fasting, which causes a marked atrophy of lymphatic organs. Subsequent feeding, which brings about the reconstitution of the tissue, is accompanied by decrease in the level of enzyme activity. From these results a relation between atrophy of the tissue and increase in β-glucuronidase activity seemed apparent.

It was therefore thought advisable to follow the behaviour of the β-glucuronidase in lymphatic tissue during atrophy induced by other experimental procedures. One of these is irradiation of the whole body, which causes in this tissue very marked regressive phenomena (De Bruyn, 1948). It was established that the enzyme increased to a maximum in the first few days after irradiation. The effect of the dose on the variation of the enzyme activity was examined as well as the effect on the period during which the activity was increased; relations between the alterations of the β-glucuronidase levels and the weight and histological structure of the tissues were also examined.

MATERIAL AND METHODS

Male albino rats weighing from 69 to 130 g. were used throughout. They were kept on a standard balanced diet (Randoin & Causeret, 1947) supplemented by green vegetables.

Conditions of irradiation were as follows: all doses of X-rays were given in single total-body exposures (140 kv, 2 mA, 3 mm. Al filter, 30 cm. target distance, 25 r./min.). Total doses administered were measured with a Victoreen 70 r. meter (Picker X-ray Corporation). Groups of four rats were irradiated simultaneously. Each animal was enclosed in an individual compartment of a wooden container, each compartment being equidistant from the centre of the field. Various doses were administered by varying the time of exposure.

From 4 hr. to 15 days after the irradiation the rats were killed by stunning and bleeding; the lymphatic organs (six superficial lymph nodes in the neck, the thymus and the spleen) were dissected out and dispersed in water (adjusted to pH 7·4) with a glass homogenizer (Potter & Elvehjem, 1936) kept in cracked ice. The final dilution of the dispersions was 1:200 (w/v). Determination of β-glucuronidase activity (with phenolphthalein glucuronide as substrate, at pH 5·2) was carried out as previously described (Pellegrino & Villani, 1956).

In some cases the dry weight of the tissue was determined by drying to constant weight in an oven at 105°.

In order to determine whether sublethal doses of whole-body X-ray irradiation altered the activity of β-glucuronidase in lymph nodes, thymus and spleen of rats, various doses of X-rays (200, 400 and 600 r.) were given to groups of animals and the enzyme activity was determined 4, 8, 12 and 18 hr. after the irradiation and later after 1, 2, 3, 5, 7, 10 and 15 days.

In order to attempt to correlate the variations observed in enzyme activity with the histological changes of the tissues after irradiation, the organs or portions of them were fixed in Bouin's fluid and embedded in paraffin wax, cut in sections 10 μ thick and stained with Carazzi's haematoxylin and eosin. Samples from at least three rats for each experimental point were examined.

RESULTS

Weight and enzyme variations in lymphatic tissues after irradiation

Lymph nodes. It is seen from Fig. 1 that after any of the three doses of X-rays the lymph nodes markedly and rapidly decreased in weight, reaching a minimum corresponding to about 50% of the initial weight between the first and the fifth day after irradiation. Eventually an increase in weight took place at a rate and to an extent which did not bear any clear relationship to the dose administered.

The activity levels of β-glucuronidase showed in every case an increase corresponding with the decrease in weight of the lymph nodes. They reached a maximum between the second and the seventh day, according to the dose of X-rays, and then returned to normal values between the fifth and the fifteenth day, according to the dose.

Thymus. The values for the thymus gland are shown in Fig. 2. The loss in weight due to irradiation was more pronounced: the minimum was reached 2 days after irradiation with 200 r. and 3 days after irradiation with 400 and 600 r. In the last two cases the organ weight was reduced to about one-fifth of the normal. A rapid increase followed with a maximum at 7–10 days for the two smaller doses, at 15 days for the larger one. The enzyme activity showed an increase, already evident 1 day after

irradiation, with a maximum 2 days after the dose of 200 r. and 3 days after the two larger doses. After 7 days the β-glucuronidase values were back to normal or even subnormal values, and no other change occurred within the experimental period.

Spleen. In the spleen also (Fig. 3) the irradiation caused a marked decrease in weight with minima at the first and second day followed by an irregular recovery. The enzyme activity, after an initial sharp increase with a maximum at the second day, showed a decrease, followed in some cases by a second increase between the seventh and the fifteenth day.

It was thought advisable to determine when the increase of the enzyme activity first became apparent. Fig. 4 shows that as early as 4 hr. after irradiation (400 r.) the organs examined undergo a loss of weight (more marked in the spleen) and in the same time show an increase in the β-glucuronidase activity (as much as 60% in the spleen).

In case variations in the water content of the tissues were concerned in the fluctuations of β-glucuronidase activity found in the first 24 hr. after irradiation, dry weights were determined in this period. No important variations in dry weight were found.

Early histological findings

The main changes in the tissues taking place within 24 hr. of total-body irradiation with 400 r. were as follows:

Lymph nodes. At 4 hr. after irradiation numerous pycnotic lymphocytes were present in the corona which surrounds the germinal centres of the lymphatic nodules and also outside in the cortex and medulla of the organs. The germinal centres showed many large phagocytes with abundant debris inside their cytoplasm. At 8 hr., pycnoses were still very evident, and phagocytosis was even more pronounced. Later the pycnotic cells were less numerous and the corona of small lymphocytes around the nodules had almost completely disappeared; at 24 hr. almost all the debris had cleared from the cells in the germinative centres. The latter were much enlarged at this stage and were composed of packed reticulo-endothelial cells with vesicular nuclei. During all this period very few mitoses could be seen.

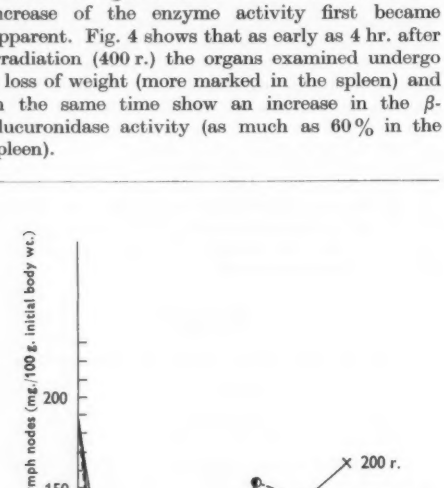

Fig. 1. Effect of total-body X-ray irradiation (200, 400 and 600 r.) on the weight and β-glucuronidase activity of neck lymph nodes of rats. Upper curves: wet weights (mg./100 g. body wt. at time of irradiation). Lower curves: β-glucuronidase activities (glucuronidase unit/g. wet wt.). For definition of glucuronidase unit (G.U.) see Pellegrino & Villani, 1956. Each experimental point corresponds to the mean of from four to eleven individual results.

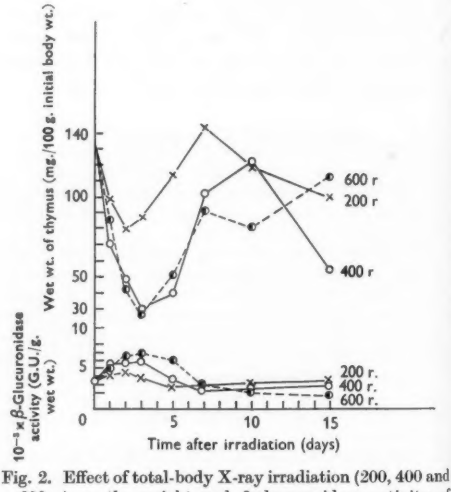

Fig. 2. Effect of total-body X-ray irradiation (200, 400 and 600 r.) on the weight and β-glucuronidase activity of thymus glands of rats. Upper curves: wet weights (mg./100 g. body wt. at irradiation). Lower curves: β-glucuronidase activities (G.U./g. wet wt.). Each experimental point corresponds to the mean of from four to eleven individual results.

Thymus. In this gland the changes were even more striking than in the lymph nodes. Pycnoses in the periphery of the thymic lobules were already so numerous at 4 hr. as to affect most of the small lymphocytes present. Pycnotic nuclei were still visible in smaller numbers 24 hr. after irradiation. In this interval debris was visible everywhere in the gland, inside and outside the phagocytes.

Spleen. Here the early visible damage was mainly localized in the lymphatic nodules, which represent, in the rat, a relatively small part of the organ. Extensive pycnosis was evident in them, as well as debris, mainly phagocytosed. In the red pulp, the haematopoietic centres underwent pycnosis and were markedly reduced at the end of the first day after irradiation.

Later histological findings

The subsequent changes from 24 hr. to 15 days in the lymphatic tissues after 400 r. can be summarized as follows.

Lymph nodes. These remained almost completely devoid of lymphocytes until the eighth day after irradiation; later, increasing numbers of these cells appeared in the cortex and in the medulla; the normal structure of the nodules had, however, not been recovered even 15 days after irradiation.

Thymus. After an extreme atrophy between the first and the fifth day after irradiation, with an almost complete disappearance of lymphocytes, these reappeared after 5 days, and their proliferation was so fast that after 10 days the thymus had almost recovered its normal appearance.

Spleen. In this organ, although atrophy of the lymphatic nodules was still evident up to 15 days, when only a small number of lymphocytes was again visible in them, in the red pulp haematopoietic tissue was again visible at 5 days and after 15 days was developed to an extent at least as great as in the normal animals.

Irradiation with 200 *and with* 600 r. As a consequence of the smaller dose of X-rays little damage was evident in the lymphatic organs, which

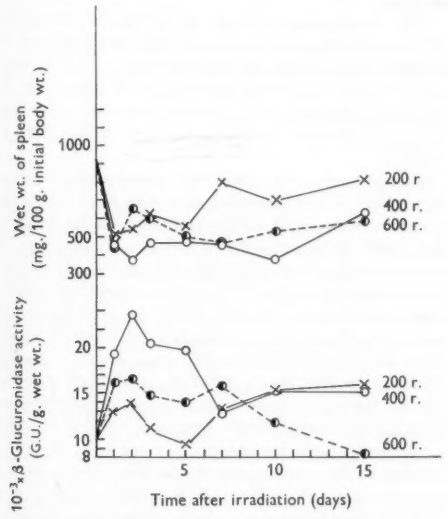

Fig. 3. Effect of total-body X-ray irradiation (200, 400 and 600 r.) on the weight and β-glucuronidase activity of the spleen of rats. Upper curves: wet weights (mg./100 g. body wt. at irradiation). Lower curves: β-glucuronidase activities (G.U./g. wet wt.). Each experimental point corresponds to the mean of from four to eleven individual results.

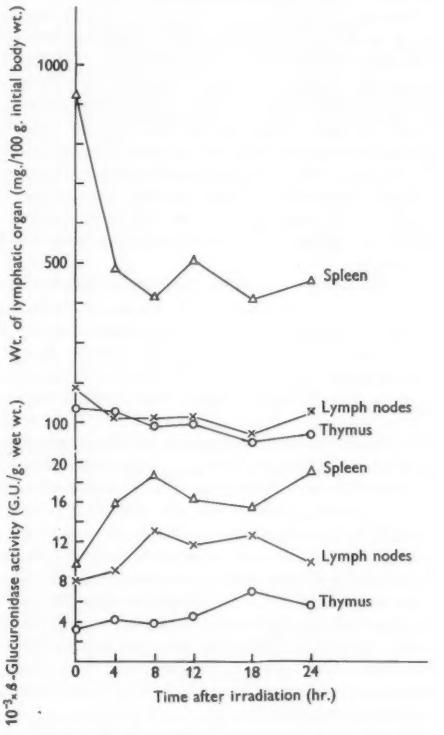

Fig. 4. Short-term effects of total-body X-ray irradiation (400 r.) on the weight and β-glucuronidase activity of lymphatic tissues of rats. Upper curves: wet weights (mg./100 g. body wt. at irradiation). Lower curves: β-glucuronidase activities (G.U./g. wet wt.). Each experimental point corresponds to the mean of from four to eleven individual results.

substantially maintained the appearance of the normal ones. On the other hand, 600 r. caused very marked lesions, with patterns similar to, but quantitatively more evident than, those after 400 r.

DISCUSSION

The results here presented demonstrate that as early as 4 hr. after X-ray irradiation an increase in the activity of β-glucuronidase is evident in lymphatic tissues (lymph nodes, thymus and spleen). The enzyme-activity levels reach eventually a maximum around the second and the third day and afterwards decline. A second less-pronounced increase in the enzyme activity can be observed later (from the seventh day onwards) in some of the organs and after some doses of X-rays.

In the first period after the irradiation the clear increase in the β-glucuronidase activity was related to the abrupt decrease in weight of the tissues (as much as 80% for the thymus). Histology showed that this period was characterized by regressive processes, which consisted mainly in extensive pycnoses of the lymphocytes, subsequent fragmentation of these cells and formation of a mass of debris, which was taken up by large phagocytes. In the thymus, where regressive processes were more evident, because of the greater proportion of lymphocytes present, this was the only period in which the activity of β-glucuronidase was clearly increased.

In the second period, in which reconstitution of the tissues took place and which extended in general from the third day to the end of the experiment, the behaviour of β-glucuronidase activity was variable. In the thymus, as soon as the regeneration of lymphocytes began, the enzyme activity fell to normal values and stayed at these levels until the end of the experiment. In the lymph nodes (Fig. 1) and in the spleen (Fig. 3) a second increase in the activity of the enzyme was evident with the doses of 200 and 400 r. but not with that of 600 r. Although these increases took place in periods of increase in weight of the organs and of regeneration of lymphocytes, no clear correlation could be found between these facts. For instance, after 600 r. the weight of the lymph nodes increased notably in the period from 7 to 10 days, coincident with the reappearance of many lymphocytes, but the β-glucuronidase activity fell in comparison with the values reached previously.

The histological examination of the tissues did not give any indication of the reason why, at 4 hr. after irradiation with 400 r., β-glucuronidase activity showed a large increase in the spleen whereas only a small increase could be observed in the lymph nodes and thymus. The fact that in the spleen the areas which showed histological damage (i.e. the lymphatic nodules) were relatively small in comparison with the red pulp, in which nothing significant could be seen at such a short interval, might indicate that at this stage the increase in enzyme activity is related to modifications which do not have an immediate histological counterpart.

Also the high levels of glucuronidase in organs almost devoid of lymphocytes as a result of the irradiation show that the main contribution to the total activity comes in these cases from other cells.

A comparison, from the data of the present and a previous paper (Pellegrino & Villani, 1956), between irradiation and fasting, both conditions in which an increase in β-glucuronidase activity could be demonstrated in lymphatic tissues, seems interesting. In both cases a rapid loss in weight of the tissues can be observed. This is more rapid after irradiation than during the fast. However, the extent of the maximum weight reduction is similar in the two conditions, being 50–55% for lymph nodes, 78–79% for the thymus and 59–70% for the spleen. The increases in the β-glucuronidase activity were similar for the lymph nodes (about 150% in both) but clearly less after irradiation for the thymus (170% after 400 r. as against 278% after 4 days of fasting) and for the spleen (228% after irradiation as against 282% after 4 days of fasting).

The mechanism of the rapid tissue involution which takes place during a short fast (for example, of 4 days in our previous experiments) seems to consist of both lymphocytolysis and of inhibition of formation of new cells, as indicated by the reduction of the mitotic rate (Frank, Kumagai & Dougherty, 1953).

After irradiation the most striking phenomena are those of lymphocytolysis, which clearly predominate; however, in the first period after irradiation with 400 and 600 r. a complete inhibition of mitoses is observed. Like the maximum increases in enzyme activity, the histological aspects of irradiated and starved tissues show some differences: more lymphocytes are present in the fasted group than in the irradiated one; phagocytosis is still going on in the former, whereas it is much reduced in the latter. However, one must conclude that histological findings fail adequately to explain the increase in β-glucuronidase activity and its singularities in the various cases. In this connexion, in the earlier paper (Pellegrino & Villani, 1956) it was demonstrated that an actual increase in enzyme activity per lymphocyte takes place during fasting, and it was also suggested that only a small proportion of the increased activity could be explained by the suppression, in the atrophic tissue, of an inhibitor of β-glucuronidase which is normally present in lymphatic tissues.

With reference to the search for the primary effects of irradiation on the tissues, the increase in β-glucuronidase activity observed seems to be the manifestation of effects not directly related to the irradiation, since it can be produced by a very different experimental condition, namely by fasting.

SUMMARY

1. Weights and β-glucuronidase activity have been determined in lymphatic organs of rats (lymph nodes of the neck, thymus and spleen) from 4 hr. to 15 days after total-body irradiation (200, 400 and 600 r.).

2. The activity of the enzyme increased in the first period after the irradiation (from 4 hr. to 5 days) when the tissues underwent a rapid decrease in weight. Histological examination showed then extensive pycnoses of lymphocytes, fragmentation of cells and phagocytosis. In the second period, in which reconstitution of the tissues took place, the behaviour of the enzyme activity was variable, depending on the organ and on the dose of radiation administered.

3. After X-ray irradiation the positive correlation between increase of β-glucuronidase activity and atrophy of the tissues, already found in the atrophy of lymphatic tissues caused by fasting, was confirmed.

4. The increase in β-glucuronidase activity observed should be considered as a secondary effect of the irradiation, as it can be reproduced by different experimental conditions such as fasting.

The authors are grateful to Professor E. Puccinelli for stimulating discussions, to Professor L. Duranti, who kindly put at their disposal the facilities of his Department for the irradiation of the rats, and to Dr R. W. R. Baker for revision of the English text. They are also indebted to Mrs Villani for the illustrations, to Mr C. Franzini and Mr P. Lemmetti for help in the preparation of the phenolphthalein glucuronide, to Mrs A. Loni for the histological slides and to Mr E. Artigiani for the care of the animals. A grant to the Department from the Consiglio Nazionale delle Ricerche, Rome, defrayed part of the expenses.

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An Improved Method of Preparing Nisin

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Nisin, produced by some strains of *Streptococcus lactis*, is one of the family of polypeptide antibiotics which has not yet been used medically (Gowans, Smith & Florey, 1952). It has, nevertheless, a practical as well as an academic interest, since it is used in the cheese-processing industry (Berridge, 1953).

The preparation of nisin has already been the subject of several papers. Mattick & Hirsch (1947) described the production of concentrates having a specific activity of two '1947 units'/μg., with a yield of 20%. Owing to the absence of adequate precautions it is only likely and not certain that the '1947 units' were almost equal to those in use now. Probably the concentrates contained about 4% of actual nisin. Concentrates made by similar methods were used for the preparation of crystalline nisin (Berridge, 1949). Losses occurred in this further purification, but they were diminished by reactivation which occurred at one stage. Since the raw material for the preparation of crystals was made commercially it was impossible to ascertain the overall yield, but it was probably of the order of 10%.

A newer method was described by Falconer (1952). In this method nisin was caused to remain with the bacterial cells by maintaining the pH at about 6. After the cells had been collected the nisin was extracted from them with acidified aqueous acetone or other solvents, and further purified by methods similar to those described earlier (Berridge, 1949). Considerable increases in yield were claimed, but no actual figures were given, and it is probable that the increases were due to the higher activity produced in the brew by techniques described at the same time elsewhere by Hirsch (1951).

The methods by which a desired substance may be concentrated and purified depend not only on the properties of the substance, but also on those of the

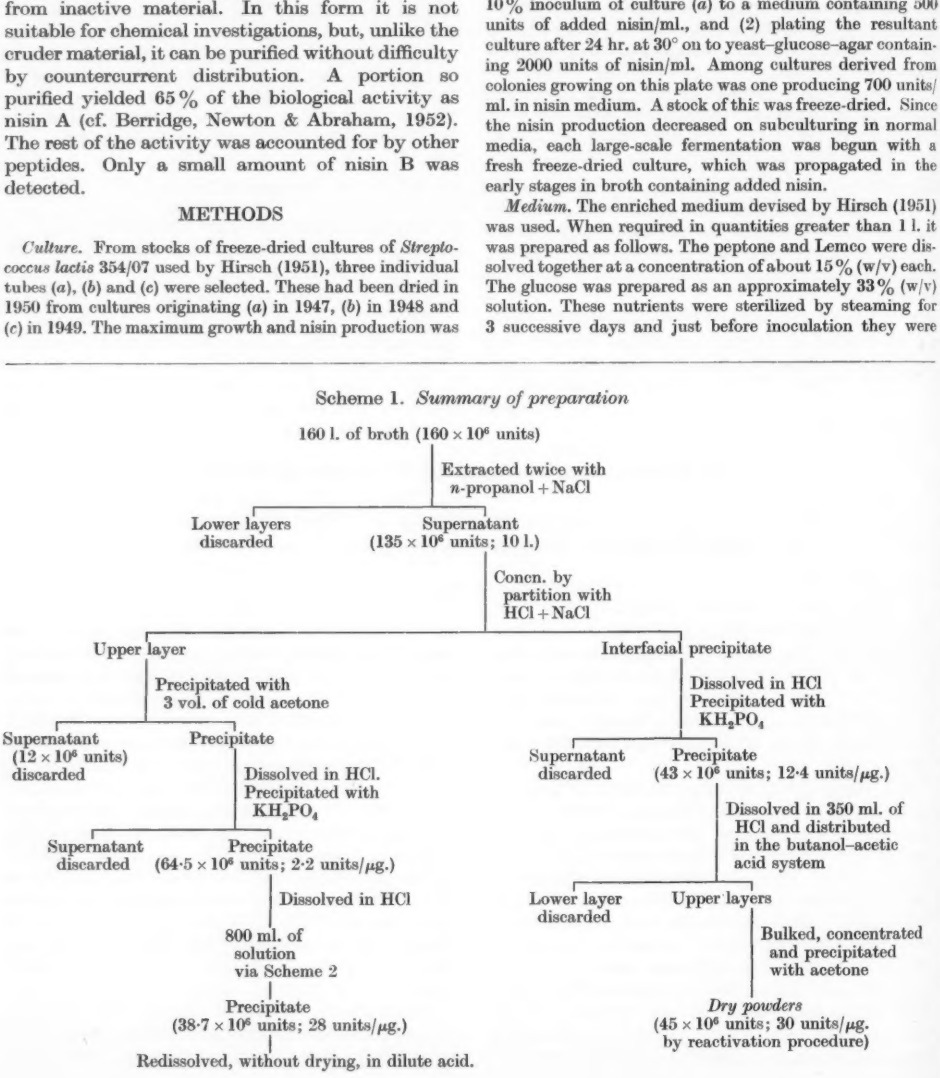
materials with which it is associated. This was so in the preparation of nisin to be described. Having taken advantage of the high-yielding medium of Hirsch and an improved method of concentration, we found the properties of the resulting mixture to be such that none of the techniques previously described (Berridge, 1949) could be used. The whole procedure was therefore worked out afresh with the result that we now have a method giving about 50 % of the brew activity as a product almost free from inactive material. In this form it is not suitable for chemical investigations, but, unlike the cruder material, it can be purified without difficulty by countercurrent distribution. A portion so purified yielded 65 % of the biological activity as nisin A (cf. Berridge, Newton & Abraham, 1952). The rest of the activity was accounted for by other peptides. Only a small amount of nisin B was detected.

METHODS

Culture. From stocks of freeze-dried cultures of *Streptococcus lactis* 354/07 used by Hirsch (1951), three individual tubes (*a*), (*b*) and (*c*) were selected. These had been dried in 1950 from cultures originating (*a*) in 1947, (*b*) in 1948 and (*c*) in 1949. The maximum growth and nisin production was obtained with culture (*a*) growing at 30° for 14 hr. in the special nisin medium (see below) which had been sterilized at 121° for 15 min. The yield was, however, low, being only 340 units/ml., compared with approximately 2000 units/ml. obtained by Hirsch. On further subculture the yield diminished to 250 units/ml. This is in agreement with the observations made by Hirsch that repeated subculture may reduce the amount of nisin a culture will produce. Following the suggestion of his paper, we suspected that intolerance to nisin was limiting the growth of the micro-organisms. Consequently we selected tolerant forms by (1) adding a 10 % inoculum of culture (*a*) to a medium containing 500 units of added nisin/ml., and (2) plating the resultant culture after 24 hr. at 30° on to yeast–glucose–agar containing 2000 units of nisin/ml. Among cultures derived from colonies growing on this plate was one producing 700 units/ml. in nisin medium. A stock of this was freeze-dried. Since the nisin production decreased on subculturing in normal media, each large-scale fermentation was begun with a fresh freeze-dried culture, which was propagated in the early stages in broth containing added nisin.

Medium. The enriched medium devised by Hirsch (1951) was used. When required in quantities greater than 1 l. it was prepared as follows. The peptone and Lemco were dissolved together at a concentration of about 15 % (w/v) each. The glucose was prepared as an approximately 33 % (w/v) solution. These nutrients were sterilized by steaming for 3 successive days and just before inoculation they were

Scheme 1. *Summary of preparation*


160 l. of broth (160 × 10⁶ units)
Extracted twice with n-propanol + NaCl
Lower layers discarded
Supernatant (135 × 10⁶ units; 10 l.)
Concn. by partition with HCl + NaCl
Upper layer
Interfacial precipitate
Precipitated with 3 vol. of cold acetone
Dissolved in HCl Precipitated with KH2PO4
Supernatant (12 × 10⁶ units) discarded
Precipitate
Supernatant discarded
Precipitate (64·5 × 10⁶ units; 2·2 units/μg.)
Dissolved in HCl
800 ml. of solution via Scheme 2
Precipitate (38·7 × 10⁶ units; 28 units/μg.)
Redissolved, without drying, in dilute acid.
Supernatant discarded
Precipitate (43 × 10⁶ units; 12·4 units/μg.)
Dissolved in 350 ml. of HCl and distributed in the butanol–acetic acid system
Lower layer discarded
Upper layers
Bulked, concentrated and precipitated with acetone
Dry powders (45 × 10⁶ units; 30 units/μg. by reactivation procedure)

mixed with the salts and tap water, which were not sterilized.

Antibiotic assay. This was carried out by the turbidimetric method previously described (Berridge & Barrett, 1952). The results were expressed in the same units.

Specific activity. The specific activity of any preparation was defined as the number of units of bacteriostatic activity/μg. of organic dry wt. The dry weights of samples were determined by evaporation at 100°, except when much salt was present. In such cases the nisin was in the form of a precipitate. This was dissolved in 0·01 N-HCl, reprecipitated with 3 vol. of acetone, again dissolved in dilute acid, and dried by evaporation at 100°. In this way sufficient separation from salt was achieved.

PREPARATIONS

Large-scale fermentation

Each culture was worked up according to the following scheme, in which arrows denote inoculation, and time intervals the periods of incubation. Hirsch's broth was used throughout, and all incubations were at 30°. Freeze-dried ampoule→10 ml. of broth, 24 hr.→100 ml., 24 hr. (nisin production and purity checked)→100 ml. containing 500–1000 units of added nisin/ml., 24 hr.→7 bottles of 100 ml., 8 hr.→all into 7 l., 8 hr. (purity checked by microscopic examination) all into 150 l. in a stainless-steel tank, incubated with pH adjustment until growth ceased. There was no agitation except during pH adjustments.

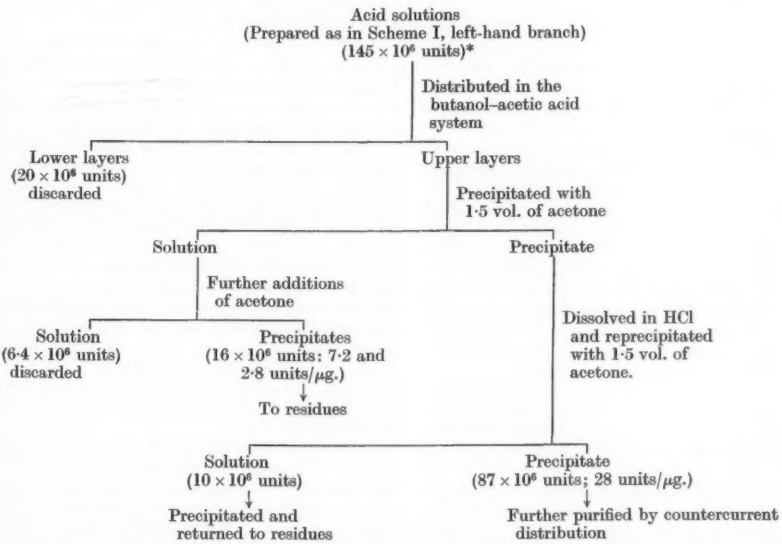
Although the medium was so highly buffered, it was thought desirable to maintain the pH between 5·9 and 6·1 by hourly adjustment. This treatment increased the growth of the streptococci as judged by opacity, but the yields of nisin did not show a marked increase over the 700 units/ml. produced by the selected culture grown without pH adjustment. Over seven fermentations the average yield was 740 units/ml., the maximum being 1000. In this medium Hirsch obtained yields around 2000 units/ml. Only once, and then on a small scale, did we observe a yield of this value.

After incubation for 16 hr. the quantity of NaOH required/hr. to maintain the pH began to diminish, and the fermentation was then stopped by bringing the pH to 1·8–2·0. This needed 6–7 litres of 10 N-HCl.

Table 1. *Calculated summary of yield of a single preparation*

	Units (millions)	Total activity (%)
Fermented acidified broth	160	100
Purified fractions (25–30 units/μg.)	83·7	52·3
Returned to residues	26·7	16·7
Discarded	12·0	7·5
Not extracted from broth	25·0	15·6
Loss during purification	12·6	7·9
	160	100

Scheme 2



* This quantity is the total of three solutions, which were derived from three fermentations and treated separately as far as the first acetone precipitates, which were then put together and refractionated as one preparation.

Preparation of nisin concentrates

The previous experience of losses of activity during preparation contrasted with the stability during countercurrent distribution and suggested that concentration by extraction with an organic solvent might be successful. Experiments on this basis led to the use of *n*-propanol (ordinary commercial grade, Honeywell and Stein Ltd.) to extract nisin from medium which was also almost saturated with NaCl.

Although most of the nisin was found to be dissociated from the cells at the end of fermentation, before acid had been added, previous experience suggested that highly acid conditions were preferable for maximum yield and stability. This procedure was therefore retained. The majority of the dead cells were allowed to settle overnight at pH 1·8–2·0, and the supernatant liquid was siphoned off. It was still cloudy, but the relatively small proportion of cells remaining was insufficient to interfere with the subsequent processes. (It was found, on the other hand, that if the settling and decanting were omitted difficulty ensued in the form of large quantities of intractable precipitate and propanol–saline emulsion, which obstinately retained the nisin. This reduced the yield by propanol extraction from nearly 100 to 30%.)

To each litre of broth was now added 100 ml. of *n*-propanol, followed by 300 g. of NaCl (commercial pure dried vacuum salt). The propanol was allowed to dissolve before the salt was added. The NaCl had the effect of diminishing the solubility of both nisin and propanol, so that the supernatant layer of propanol which appeared after a short time carried most of the activity. This layer was next separated by siphoning and centrifuging, and a second extraction was made with 30 ml. of propanol/litre of residual broth.

In this way 85–100% (the mean of six experiments was 93·5%) of the nisin from 150 l. of broth was concentrated into 10 l. of clear-yellow propanolic solution. (Both layers, of course, contained propanol, but 'propanolic' is used here and subsequently to denote the supernatant solution, which is richer in propanol.)

The propanolic solution was further concentrated by a repetition of the process used in its preparation; to each litre was added 7 l. of 0·1 N-HCl, followed by excess of NaCl, whereby all the nisin and some of the propanol were salted out. After about 1 hr. the mixture separated into three layers, the upper two being of small volume. After collecting and centrifuging the supernatant layers, one-half of the nisin was found in the clear-brown top layer, and the rest in a grey precipitate which formed a layer between the propanol and the aqueous NaCl solution. The propanolic solution and the precipitate, which was separated and redissolved in 0·05 N-HCl, were purified separately.

(1) *The propanolic solution.* The process of salting-out could be repeated with the concentrated propanolic solution, to achieve further concentration, but whenever this was done marked losses in antibiotic activity were experienced. A different method was therefore used for further concentration. The propanolic solution was cooled to 0°, precipitated with 3 vol. of ice-cold acetone, and the mixture cooled in metal centrifuge buckets to −15° immediately before centrifuging for 10 min. Even after this time the mixture was quite cool, and when the precipitate was redissolved at once, in the minimum of 0·05 N-HCl, full recoveries were obtained. Previous experiments had shown that without the precaution of cooling much nisin was lost, although nisin itself is not inactivated by acetone.

In order to assist solution of the nisin and at the same time to destroy any enzymes which might have been present, the mixture of precipitate and 0·05 N-HCl was heated at 70° for 30 min. Traces of insoluble substances remaining were separated by centrifuging and they were found to have only negligible activity.

To the warm acid solution was added KH_2PO_4 to a concentration of M. Nisin then began to be precipitated and the process was allowed to go to completion overnight at 1–4°. The precipitate, after centrifuging, was dissolved in 0·05 N-HCl to give a nisin solution with a specific activity of about 2·2 units/μg. The yields here and at subsequent stages are shown in Schemes 1 and 2 and in Table 1.

Further purification was achieved by partition in a mixture of *n*-butanol–water–acetic acid (3:4:1, by vol.) and enough concentrated ammonia to bring the lower phase to pH 3·5. The partition coefficient [=(concn. in butanol phase)/(concn. in aqueous phase)] of nisin increases with increasing pH, but the solubility diminishes. A convenient compromise is reached at pH 3·5. Here the nisin has a partition coefficient of about 4, whereas the overall coefficient of the bulk of the solutes is about 0·5. The solution of nisin in 0·05 N-HCl served in place of water for the first partition. Two more extractions of the remaining lower layer were made with upper layer prepared separately. These extractions should have removed practically all the bacteriostatic activity from the aqueous phase, but in fact only 80–90% of it was finally obtained in the butanol phase.

Owing to the low solubility of nisin in this system it was necessary to use upper phase equal in volume to that of the lower phase for each extraction. There was thus a threefold increase in volume at this stage.

Nisin was separated from the mixed upper layers by partial precipitation with acetone in the cold, the technique described above being used. Precipitates were collected after 1·5, 2 and 6 vol. of acetone had been added. The last two precipitates were low both in quantity and in specific activity, so that only the first was chosen for further purification. It was dissolved in the minimum of 0·01 N-HCl and heated to boiling point for 1 min. Fractionation with acetone by the same scheme was now repeated. The results here were a little different owing to the absence of the butanol, but the first precipitate still contained the major part of the nisin and had the highest specific activity, namely 28 units/μg. The nisin A separated by countercurrent distribution by Berridge *et al.* (1952) had a specific activity of 32 units/μg. It was therefore reasonable to assume that the present preparation was approaching purity. It was redissolved in 0·01 N-HCl and again boiled for 1 min., for partial sterilization, and stored in the refrigerator until it could be further purified by countercurrent distribution.

(2) *The interfacial precipitate.* The solution in HCl (see above) was purified by precipitation from M-KH_2PO_4 as described for the propanolic solution. Since the specific activity of the precipitate was much higher in this case, only the fractionation with butanol was carried out and further fractionation with acetone was not necessary. Two-thirds of the butanol were evaporated under reduced pressure at a bath temperature of 47–50°, and the nisin was

separated by the addition of excess of acetone (6 vol.). In this case the precaution of cooling the mixture was not necessary, and when the precipitate, moist with acetone, was immediately redissolved in dilute acid, there were no losses in activity. This was the usual experience with precipitates having a specific activity of about 25 units/μg. or more, and was in marked contrast to the instability of the impure products at earlier stages of the preparation. This production of a precipitate containing 25–30 units/μg. completed the preparation of the second fraction.

RESULTS

A particular example of the preparation is given in Schemes 1 and 2. The products of three batches were bulked for the preparation described in Scheme 2, and the percentage recovery has been used to calculate the yield for this subsection of Scheme 1, so as to arrive at a total yield for a single example beginning with 160×10^6 units. It can be seen that over 50 % of the activity of the broth was obtained in a highly purified form.

Countercurrent distribution (340 transfers) of the first fraction (i.e. that derived from the propanolic solution) in *n*-butanol–ammonium acetate–acetic acid buffer showed one major and several minor peaks, and permitted the isolation of the material of the major peak in a yield of about 65 % of the total biological activity submitted to the distribution. About 20 % of the activity remained with the lower layers at the beginning of the distribution series where it was associated with a small amount of insoluble material and stable emulsion. The rest of the activity was accounted for by various minor peaks and small portions of the major peak which were overlapped by impurities. A portion of the isolated material was submitted to analytical countercurrent distribution in the solvent used by Berridge *et al.* (1952). The results confirmed that the fraction behaved as a single peptide. It had the same partition coefficient and the same specific activity as nisin A.

Fraction 2 has not yet been subjected to countercurrent distribution, but the acetone precipitates have been dried, some by freeze-drying an aqueous solution, some by washing with dried acetone and removing acetone *in vacuo* at $-5°$. Such powders do not give the expected activity on dissolving in 0·01 N-HCl. Full recovery of activity has been obtained, however, in some cases, e.g. in fraction 2 of fermentation no. 6, by dissolving in a small amount of 0·01 N-HCl, adding ethanol to 80 % (v/v), and leaving at room temperature for 4–5 hr., before diluting and assaying. A similar recovery of activity can be obtained by using a solution of 6 M urea in the place of 80 % ethanol. Partial reactivation is also obtained by leaving a dilute solution in 0·01 N-HCl standing at room temperature for 24 hr. before assaying, and again by heating the dilute solution to 100° for 1 min. Since these observations the drying of nisin has been avoided until the phenomena are better understood.

DISCUSSION

The stability of nisin under certain conditions (for example, it can be boiled in 0·05 N-HCl for several minutes without its activity being diminished) is in marked contrast to its lability during isolation. In attempting to concentrate solutions in aqueous propanol beyond a certain level, in trying to extract nisin from broth with butanol and in exploring the possibilities of fractional precipitation with acetone at room temperature, severe losses were encountered. The same occurred when light petroleum was added to a mixture of the propanolic solution and dilute acid with the aim of transferring the nisin to the aqueous phase. The losses in preparing nisin by the method of adsorption on to chloroform emulsion were also heavy (Mattick & Hirsch, 1947). All these methods had one, possibly two, features in common; they produced a copious precipitate or emulsion and they denatured any sensitive proteins which may have been present, either by the action of the solvent itself or by adsorption on to the surface of the emulsion. A quantity of material which remained insoluble in dilute acid was always formed when these methods were applied. The readiness with which proteins form complexes during denaturation is well known (see, for example, Kleczkowski, 1949; Turner & Boyer, 1952), and it is reasonable to assume that low yields of nisin by these methods were due to the irreversible formation of complexes between the nisin and proteins derived from the bacteria. Hence the need for a different technique in preparing nisin from an enriched medium.

Results obtained in the course of this work confirmed the earlier observation (Berridge, 1949) that the use of phosphate to salt out solutions made from dried crude nisin gives a variable (40–200 %) increase in activity. For this reason the phosphate salting-out step was retained, but, it is to be noted, at a different pH.

The failure of the butanol mixture, in which the partition coefficient of the nisin was 4, to extract more than 90 % of the bacteriostatic activity from the lower layer with three equal volumes of upper layer suggests the presence of small quantities of an antibiotic of much lower partition coefficient. (The theoretical yield for three such extractions is 99·2 %.)

Although the yield of purified material is between 50 and 60 % it is worth emphasizing that most of the rest of the active material can be accounted for in the form of less pure fractions, and thus that the nisin

has not actually been destroyed. It would be available for uses in which chemical homogeneity was not essential.

SUMMARY

1. A method of preparing the antibiotic nisin by extraction with solvents from broth in which the special strain of *Streptococcus lactis* has grown is described.

2. The nisin was purified by salting out, solvent extraction and fractional precipitation with acetone.

3. The yield of almost pure antibiotic was about 1 g. from each 80 l. of broth.

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